AD_____

Award Number: DAMD17-97-1-7151

TITLE: Regulation of Sialomucin Complex Expression and Its Affect on Her Receptor Interaction (96 Breast)

PRINCIPAL INVESTIGATOR: Shari Price-Schiavi

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33136

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (15 Aug 98 -14 Aug 99)

4. TITLE AND SUBTITLE

Regulation of Sialomucin Complex and Its Effect on HER Receptor Interaction

5. FUNDING NUMBERS

DAMD17-97-1-7151

6. AUTHOR(S)

Shari A. Price-Schiavi

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Miami School of Medicine
Miami, Florida 33136

e-mail:

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release: distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit, ASGP-2, which can act a s a ligand for the receptor tyrosine kinase ErbB2. SMC is a highly expressed protein on the surface of ascites 13762 rat mammary adenocarcinoma cells, approximately 10^2 times the level in lactating mammary gland and 10^4 times that in virgin mammary gland. SMC is largely post-transcriptionally regulated in developing mammary gland. Additionally, SMC can be post-transcriptionally in normal cultured rat mammary epithelial cells (MEC) but nor 13762 MAT-B1 mammary tumor cells by Matrigel by inhibition of SMC precursor synthesis. SMC is also post-translationally regulated by TGFβ in cultured normal MEC by inhibition of SMC precursor processing into mature ASGP-1 and ASGP-2. Interestingly, SMC levels in 13762 MAT-B1 tumor cells are unaffected by TGFβ. SMC and ErbB-2 have similar expression patterns in normal developing mammary gland and can be found in a complex in virgin as well as lactating mammary tissue. Thus, SMC is regulated by two novel post-transcriptional mechanisms in normal mammary epithelial cells but not 13762 tumor cells and its complex formation with the receptor ErbB-2 may play some role in mammary development and differentiation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer; Sialomucin Complex; Gene Expression; ErbB Receptor; Mammary Epithelial Cells; Growth Factor	41
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

~~NA~~ SS In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

_NA_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_✓_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_NA_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_NA_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature

9.10.99
Date

Table of Contents

Introduction

Sialomucin complex (SMC) was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (Sherblom and Carraway, 1980). The complex consists of a peripheral, O-glycosylated mucin subunit ASGP-1 (Sherblom *et al.*, 1980a,b), and an N-glycosylated integral membrane glycoprotein ASGP-2 to which ASGP-1 is tightly, but non-covalently, bound (Sherblom and Carraway, 1980; Hull *et al.*, 1990). Recent studies have demonstrated that SMC is the rat homolog of human MUC4 (Moniaux *et al.*, 1999). Several studies suggest that the two-subunit SMC is a multi-functional glycoprotein complex. Overexpression of SMC can provide anti-recognition and anti-adhesive properties to tumor cells (Komatsu *et al.*, 1997). Furthermore, SMC expression in tumor cells reduces their killing by natural killer cells (Komatsu *et al.*, 1999). ASGP-2 has two epidermal growth factor-like domains, which have all of the consensus residues present in active members of the epidermal growth factor family (Sheng *et al.*, 1992). Moreover, SMC has been shown to bind to and modulate phosphorylation of the receptor ErbB2 (Carraway *et al.*, 1999). Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the epidermal growth factor family of receptors via its interaction with ErbB2 (Carraway *et al.*, 1999 Carraway *et al.*, 1992). This interaction may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (Juang *et al.*, 1996) and the rapid growth of these cells *in vivo*. Sialomucin complex expression has been described in a number of normal secretory epithelial tissues in the adult rat including mammary gland (Rossi *et al.*, 1996; McNeer *et al.*, 1997) and appears to have multiple and complex regulatory mechanisms. Because overexpression of SMC may lead to deleterious consequences, it is important to understand how expression of this protein is regulated as well as the consequences of its interaction with ErbB-2. Thus, we are characterizing regulation of SMC expression in normal mammary epithelial cells and 13762 mammary ascites tumor cells. Furthermore, we are characterizing the interactions ASGP-2 and ErbB-2 and the downstream results of this interaction. For this report we describe the regulation of SMC by TGFβ in cultured primary mammary epithelial cells and tumor cells. TGFβ inhibits induction of SMC expression when the cells are put into culture and is a rapid effect. The presence of TGFβ does not affect the ratio of membrane-bound to soluble form of SMC produced, nor does it affect the rate of SMC turnover in these cells. Unlike Matrigel, which inhibits SMC precursor synthesis (Price-Schiavi *et al.*, 1998), TGFβ has no effect on SMC precursor synthesis. Instead, TGFβ alters the processing of SMC precursor into mature SMC (ASGP-1/ASGP-2), a novel TGFβ action which appears not to be a consequence of the effects of TGFβ on transcription. In addition, we describe characterization of ASGP-2 and ErbB-2 complex formation in normal rat mammary epithelial cells. Both SMC and ErbB-2 are developmentally regulated in normal rat mammary tissue, and the two proteins form a complex in virgin as well as lactating mammary tissue. Although SMC and ErbB-2 have similar expression patterns in developing mammary tissue, they have different regulatory mechanisms in cultured mammary epithelial cells. Thus, we propose to use 13762 MAT-B1 cells to further characterize ASGP-2/ErbB-2 interactions.

Regulation of SMC expression in normal cultured rat mammary epithelial cells

SMC expression in cultured mammary epithelial cells (MEC) in the presence or absence of TGFβ-We have shown previously that SMC/Muc4 protein is induced rapidly when isolated mammary epithelial cells are cultured as a monolayer on plastic tissue culture dishes. Further, we demonstrated that TGFβ post-transcriptionally regulates SMC in these cells (Price-Schiavi *et al.*, 1998; see attached reprint). The aim of these studies was to define the mechanism for post-transcriptional regulation of SMC by TGFβ. A time course was performed to characterize the expression pattern of SMC in the presence or absence of TGFβ in cultured MEC. Isolated MEC from virgin rats were cultured on plastic in Ham's F-12 medium supplemented with insulin/transferrin/selenium and 1 mg/ml BSA with or without 200 pM TGFβ. Cells were harvested at times ranging from 0 to 24 hr after plating and lysed, and total protein was quantified. SMC content was analyzed by immunoblotting with mAb 4F12. In the absence of TGFβ, SMC appears at about 5 hr after plating and reaches maximal levels only after 24 hr (Fig. 1). In the presence of TGFβ, SMC also appears at about 5 hr after plating but levels off by about 12 hr. The maximal level of SMC in MEC cultured in the presence of TGFβ is about 50% of that in cells cultured without TGFβ.

TGFβ inhibits the growth of many epithelial cell types; therefore, SMC inhibition by TGFβ may be due to differences in cell number after cells are treated with the growth factor. This issue was controlled by carefully loading equivalent amounts of total protein for immunoblot analysis. However, to confirm that this is not a cell number effect, virgin MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with ITS (insulin, transferrin, and sodium selenite) and 1 mg/ml BSA in the presence or absence of 200 pM TGF-β. After 48 hours, half the cells were harvested and lysed. Total protein was quantified by Lowry assay and 5 μg total protein was loaded for immunoblot analysis. The other half of the cells was harvested using an enzyme-free cell dissociation buffer (Gibco) and counted on a hemacytometer. An equivalent number of cells (5.0×10^4 cells/lane) were loaded for immunoblot analysis. The inhibition of SMC levels by TGF-β is unaffected by cell number (Fig. 2). In fact when equivalent numbers of cells are loaded for the immunoblots, the inhibition is more pronounced than when equivalent amounts of total protein are analyzed.

The specificity of the TGF-β effect was studied by the addition of a neutralizing antibody to TGF-β. MEC were cultured in the presence or absence of 200 pM TGF-β. 30 μl of anti-TGF-β antibody was either incubated with the TGF-β for 30 min at 4°C prior to addition to the culture or the antibody was added to the culture at the time of plating. After 24 hr the cells were analyzed by immunoblotting. The neutralizing antibody inhibited the TGF-β effect on SMC levels when it was incubated with the TGF-β prior to addition or when it was added to the culture at the time of plating (Fig. 3). These data indicate that the inhibition of SMC expression by TGF-β is specific.

TGFβ can reduce SMC levels in cultured MEC in less than 24 hr, suggesting that this is a rapid response. To determine more accurately how fast TGFβ can reduce SMC levels, MEC were cultured for 24 hr to induce high levels of SMC. TGFβ was then added to a final concentration of 200 pM to half the cells, and samples were harvested 6 and 24 hr later for immunoblot analyses. SMC expression was inhibited by TGFβ within 6 hr of its addition (Fig. 4A); the inhibition was more pronounced 24 hr after addition of

TGFβ. The relatively rapid effects suggest that new transcription and protein synthesis are not necessary for TGFβ mediated depression of SMC levels. To test this idea, MEC from virgin rats were cultured for 24 hr, then 200 pM TGFβ and/or 10 μg/ml cycloheximide were added to the media. Cells were harvested after 6 hr for immunoblot analyses with mAb 4F12. As demonstrated previously, SMC levels were reduced by TGFβ within 6 hr of its addition. The presence of cycloheximide, which inhibits new protein synthesis, did not reverse reduction of SMC levels by TGFβ (Fig.4B), indicating that no new protein synthesis is required for TGFβ to reduce SMC levels.

Effect of TGFβ on the production of soluble SMC--Normal mammary tissue produces both soluble and membrane forms of SMC in a ratio of ~60% membrane:40% soluble form (Rossi *et al.*, 1996). One possible effect of TGFβ is alteration of the ratio of membrane bound to soluble form of SMC by stimulating conversion of the membrane precursor to soluble form. Thus, in the presence of TGFβ, the detectable SMC in the cell would be reduced because it would be secreted from the cell. To test this possibility, MEC were cultured in the presence or absence of 200 pM TGFβ for 48 hr and lysed in RIPA buffer. The lysates were sequentially immunoprecipitated twice with anti-C-Pep, a polyclonal antibody which recognizes an epitope in the C-terminal (cytoplasmic) domain of SMC, and once with polyclonal anti-ASGP-2. Two rounds of immunoprecipitation with anti-C-pep will clear the cell lysate of membrane bound form of SMC (Rossi *et al.*, 1996), while the anti-ASGP-2 recognizes the remaining SMC, the soluble form. Immunoprecipitates were analyzed by immunoblotting with mAb 4F12, which recognizes both membrane and soluble SMC (Rossi *et al.*, 1996). The presence of TGFβ does not affect the ratio of membrane to soluble form (Fig. 5A). Both treated and untreated cells produce ~ 55% membrane bound: ~ 45% soluble form (Fig. 5B), and soluble form SMC was detected in the conditioned media from both treatment groups. The only difference was that the overall level of SMC produced in the TGFβ treated cells was lower than that produced in untreated cells. These data rule out the possibility that the apparent decrease in SMC levels in the cultured MEC is due to a shift of membrane SMC to the soluble form.

Effect of TGFβ on turnover of SMC– Since the effect of TGFβ on SMC expression is rapid, another potential mechanism for its repression is the acceleration of SMC turnover. Recently, we have observed that SMC expression can be completely blocked by tunicamycin, an inhibitor of N-glycosylation (Elbein, 1984). We have used this synthesis block to investigate the turnover of SMC. When normal MEC are treated with tunicamycin for 24 hr, SMC becomes undetectable (Zhu, X., unpublished). Since the transmembrane subunit of SMC (ASGP-2) is N-glycosylated, we hypothesize that inhibition of N-glycosylation blocks its proper folding and leads to degradation of newly synthesized SMC. To determine if TGFβ increases the turnover of SMC, MEC were cultured for 24 hr, and TGFβ and tunicamycin were added at final concentrations of 200 pM and 1 μg/ ml, respectively. Samples were harvested at times ranging from 0 to 20 hr after addition of these agents and analyzed by immunoblotting with mAb 4F12. The resulting bands were quantified by densitometry (Figs. 6A and B). The presence of TGFβ did not cause an increased reduction in SMC levels in the tunicamycin-treated cells. These data suggest that the mechanism for the effect on SMC expression by TGFβ does not involve alteration of the rate of SMC turnover in normal cultured MEC.

Biosynthesis of SMC in the presence or absence of TGFβ–To investigate the effect of TGFβ on SMC translation, a labeling experiment was performed. MEC were cultured for 24 hr, at which time half the cells were treated with 200 pM TGFβ. After an additional 24 hr, the cells were labeled for times ranging from 0 to 6 hr with [^{35}S]Cys +[^{35}S]Met. Cells were harvested, lysed, and immunoprecipitated with anti-ASGP-2 polyclonal antibody, which recognizes both the SMC precursor and mature ASGP-2. Samples were subjected to SDS-PAGE and fluorography. Total protein synthesis was similar in both treated and untreated samples, indicating that TGFβ did not inhibit total protein synthesis. The amount of accumulating SMC precursor detected in both treated and untreated samples was similar for all time points (Fig. 7A). Precursor accumulation relative to total labeled protein was quantified by densitometry (Fig. 7B). These data demonstrate that similar levels of precursor were synthesized in the presence or absence of TGFβ, indicating that TGFβ does not affect the rate of SMC precursor biosynthesis (message translation). Thus, the reduction of SMC levels by TGFβ involves a different mechanism from that for Matrigel, which inhibits SMC precursor biosynthesis (Price-Schiavi *et al.*, 1998, see attached reprint).

Effect of TGFβ on processing of SMC precursor--Since TGFβ does not affect SMC translation or the turnover of the mature protein, another possibility is that TGFβ could affect the processing of the SMC precursor into mature ASGP-1/ASGP-2. In order to test this possibility, a pulse-chase experiment was performed. MEC were cultured 24 hr, and TGFβ was added to half the cells to a final concentration of 200 pM. After an additional 24 hr, the cells were pulse-labeled for 30 min with [^{35}S]Cys +[^{35}S]Met. Following the pulse, the cells were washed in prelabeling medium twice and incubated in chase medium for times ranging from 1 to 8 hr. TGFβ was present in half the samples at a concentration of 200 pM throughout the labeling procedure. After the chase, cell lysates were immunoprecipitated with anti-ASGP-2 antibodies. Immunoprecipitates as well as an aliquot of non-immunoprecipitated cell lysate were subjected to SDS-PAGE and fluorography. Total labeled protein was similar for both samples with and without TGFβ, suggesting that protein synthesis is not inhibited by TGFβ in these cells (Fig. 8A). The level of SMC precursor is similar for treated and untreated samples, again suggesting that TGFβ does not inhibit the translation of SMC (Fig. 8A). To determine whether TGFβ affects processing of SMC precursor into mature SMC, the bands for SMC precursor and mature ASGP-2 were quantified by densitometry, and the ratios of SMC to mature ASGP-2 for each time point were plotted. In the absence of TGFβ SMC precursor is processed rapidly into mature ASGP-2. In fact, mature labeled ASGP-2 is detected in the pulse sample, and >50% of the precursor is processed into mature ASGP-2 in one hour (Fig. 8B). In the presence of TGFβ, the SMC precursor is processed more slowly; after 4 hr only about 20% of SMC precursor had been processed into mature SMC. In addition, much less mature ASGP-2 accumulates in the TGFβ samples. These data indicate that TGFβ affects the processing of the SMC precursor into mature SMC, causing the apparent reduction in SMC levels when cells are cultured in the presence of TGFβ. Once again, these data point to a different mechanism of post-transcriptional regulation of SMC from that with Matrigel, which occurs by a reduction in SMC precursor synthesis.

Effect of TGFβ on SMC levels in cultured 13762 MAT-B1 ascites cells—Because few of the exogenous factors we tested had any effect on SMC levels in normal cells (Table 1, data discussed in previous progress report), we did not test them in 13762 MAT-B1 tumor cells. Thus, for Statement of Work Task 5, we chose instead to test only those factors that had a significant effect on SMC levels in the normal mammary epithelial cells (i.e. Matrigel and TGFβ). SMC levels in 13762 MAT-B1 tumor cells are unaffected by culture in Matrigel (Price-Schiavi *et al.*, 1998; see accompanying reprint), suggesting that this level of regulation has been disrupted in these cells. To determine if the 13762 MAT-B1 cells are also refractory to TGFβ, MAT-B1 cells were cultured on plastic in DMEM medium supplemented with 10% FCS in the presence or absence of TGFβ for 24 hours. Cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. SMC levels in the cultured MAT-B1 tumor cells were similar in the presence or absence of TGFβ (Fig. 9). This result suggests that in addition to loss of regulation by Matrigel, SMC regulation by TGFβ is also defective in these cells. This represents a second level of disruption of SMC regulation in the 13762 ascites tumor cells. Thus, regulation of SMC in 13762 ascites tumor cells is disrupted at multiple levels including gene amplification (Wu *et al.*, 1992), overproduction of SMC transcript (Price-Schiavi, unpublished), and loss of posttranscriptional regulation by ECM (Matrigel) and TGFβ.

ASGP-2 and ErbB-2 interactions in mammary epithelial cells

The last part of these studies involves characterizing the interactions of ASGP-2 and ErbB-2 and downstream signaling consequences in normal mammary epithelial cells. ASGP-2 has been shown to bind to and modulate phosphorylation of ErbB-2 in 13762 MAT-C1 ascites tumor cells. To compare the expression patterns of SMC and ErbB-2 in normal developing mammary tissue, a time course was performed. Mammary tissue homogenates from virgin, mid- and late- pregnant, lactating, and involuting mammary tissue were immunoprecipitated with either anti-ASGP-2 or anti-ErbB-2 polyclonal antibodies. Immunoprecipitated proteins were analyzed by immunoblot with either anti-ASGP-2 mAb 4F12 or anti-ErbB-2 monoclonal antibodies. Both SMC and ErbB-2 are present at low levels in virgin gland, increase during pregnancy reaching maximal levels during lactation, and decline significantly during involution (Fig. 10). These data suggest that the expression patterns for SMC and ErbB-2 are similar *in vivo* and support the idea that the two proteins may form a complex in normal rat mammary tissue.

We have previously demonstrated that ASGP-2 and ErbB-2 can be co-immunoprecipitated from lactating mammary tissue. To determine if there are major changes in ASGP-2/ErbB-2 complex formation during mammary development and differentiation, co-immunoprecipitations were performed from both virgin and lactating mammary tissue. Whole mammary tissue was solubilized and immunoprecipitated with either anti-ASGP-2 or anti-ErbB-2 polyclonal antibodies, or nonimmune rabbit serum. Immunoprecipitated proteins were subjected to immunoblot analysis with anti-ASGP-2 or anti-ErbB-2 monoclonal antibodies. ASGP-2 was immunoprecipitated with anti-ErbB-2, and ErbB-2 was immunoprecipitated with anti-ASGP-2 (Fig. 11) lactating mammary tissue as expected. ASGP-2 and ErbB-2 were also co-immunoprecipitated in virgin mammary tissue. Neither ASGP-2 nor ErbB-2 was detected in the nonimmune rabbit serum control immunoprecipitations. These data suggest that as in the 13762 MAT-C1

cells, SMC (ASGP-2) and ErbB-2 form a complex in both virgin and lactating normal rat mammary tissue. In addition, these data suggest that SMC and ErbB-2 form a complex during several stages of mammary development.

To determine if SMC also forms a complex with ErbB-3, a similar co-immunoprecipitation was performed. Lactating mammary gland lysates were immunoprecipitated with either polyclonal anti-ASGP-2, anti-ErbB-2, anti-ErbB-3, or nonimmune rabbit serum. Immunoprecipitated proteins were subjected to immunoblot with anti-ASGP-2 mAb 4F12. ASGP-2 and ErbB-2 were co-immunoprecipitated, but ErbB-3 was not (Fig. 12). This result is in agreement with reported data from an insect cell system that demonstrated that ASGP-2 could form a complex with ErbB-2 but not ErbB-3. Thus, this result suggests that ASGP-2 and ErbB-3 do not form a complex in normal lactating mammary tissue.

In order to characterize ASGP-2/ErbB-2 interactions in normal cultured mammary epithelial cells, it is necessary to establish culture conditions favorable for reasonable expression of both SMC (ASGP-2) and ErbB-2. SMC expression is highest when MEC are cultured on plastic in the presence of fetal calf serum, while culture of MEC in Matrigel (which mimics the *in vivo* state for growth of MEC) substantially inhibits SMC levels (Price-Schiavi *et al.*, 1998, see accompanying reprint). In order to determine if ErbB-2 expression followed the same pattern as SMC, MEC were cultured either on plastic or embedded in Matrigel in the presence or absence of 10% fetal calf serum. Unfortunately, the expression pattern of ErbB-2 in normal cultured MEC is very different from that of SMC. ErbB-2 is maximally expressed when MEC are cultured embedded in Matrigel (SMC levels are very low under these culture conditions), while it is almost undetectable when MEC are cultured on plastic where SMC levels are highest (Fig. 13).

This discrepancy in the *in vitro* expression patterns of SMC and ErbB-2 brings some difficulty to the experiments outlined for Tasks 6 and 7 in the Statement of Work for characterizing the ASGP-2/ErbB-2 interactions and downstream consequences. However, the studies outlined in Tasks 6 and 7 can be carried out in cultured 13762 MAT-B1 tumor cells, which produce both SMC and ErbB-2 when the cells grown in short term culture. Thus, we have chosen to pursue these studies with this cell line instead of the normal primary mammary epithelial cell cultures, and these studies are currently under way.

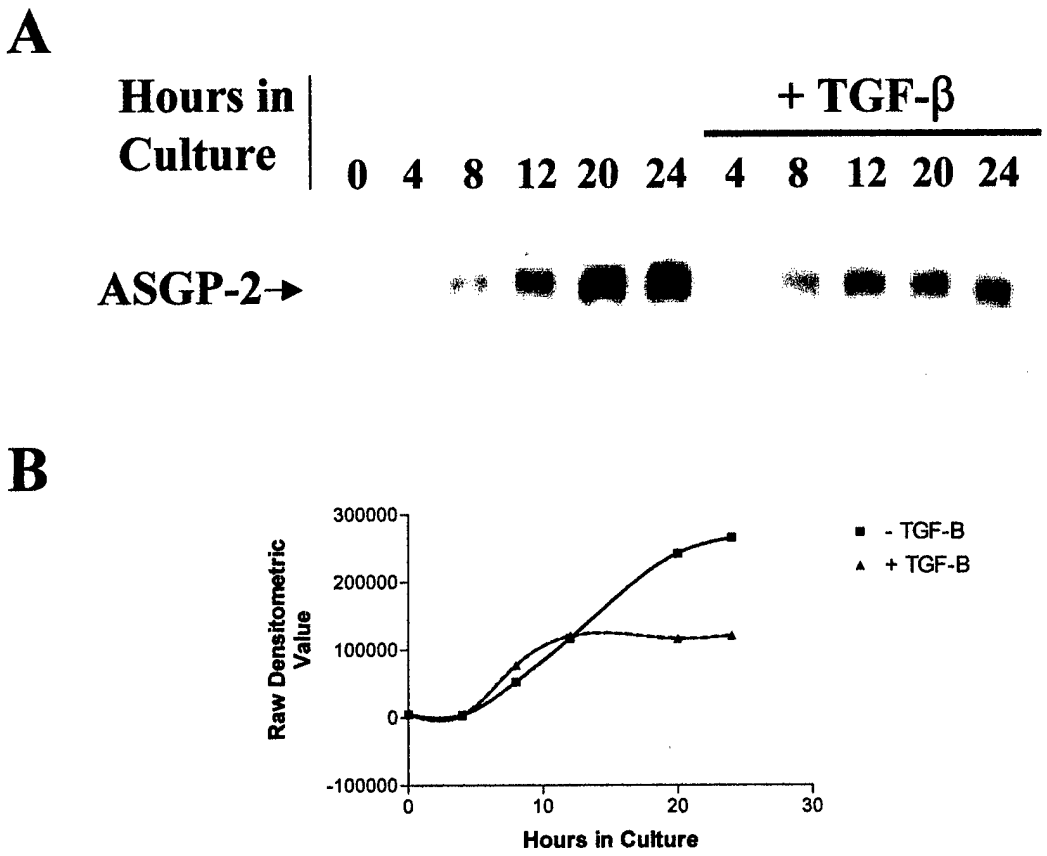


Fig. 1. Effect of TGF-β on SMC induction in normal cultured MEC. Virgin MEC were isolated and plated in the presence or absence of 200 pM TGF-β. Samples were harvested at various times after plating as indicated at the top of the figure. 5 μg total protein were analyzed by immunoblot analysis with mAb 4F12. A) Western blot of time course of SMC expression in the presence or absence of TGF-β. B) Plot of time course of SMC expression in the presence or absence of TGF-β. The bands from A) were quantified by densitometry and the results were plotted.

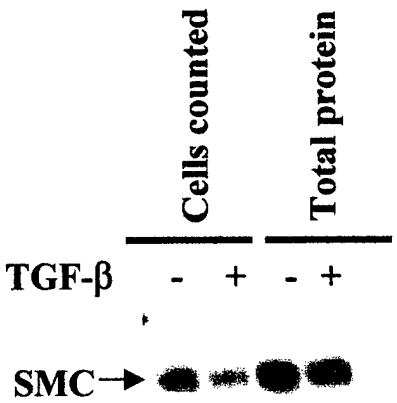


Figure 2. TGFβ repression of SMC synthesis is not and effect of cell number. Virgin MEC were isolated and cultured in the presence or absence of 200 pM TGFβ for 48 hours. Cells were harvested and either 5 μg total protein or 5.0 x 10^4 cells were loaded for immunoblot analysis with mAb 4F12 as indicated at the top of the figure.

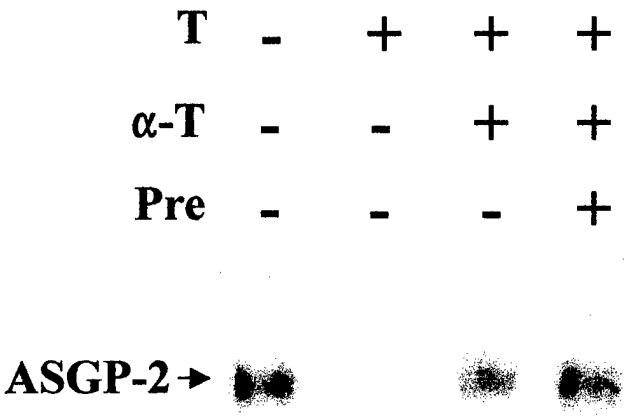


Figure 3. A neutralizing antibody to TGFβ reverses TGFβ induced SMC synthesis in cultured normal MEC. Virgin MEC were cultured in the presence or absence of 200 pM TGFβ and anti-TGFβ neutralizing antibody for 24 hours. Cells were harvested, and 5 μg total protein were subjected to immunoblot analysis with mAB 4F12. Abbreviations: T, TGFβ; α-T, anti-TGFβ neutralizing antibody, Pre, pre-incubation of anti-TGFβ neutralizing antibody with TGFβ for 30 min prior to addition to the culture.

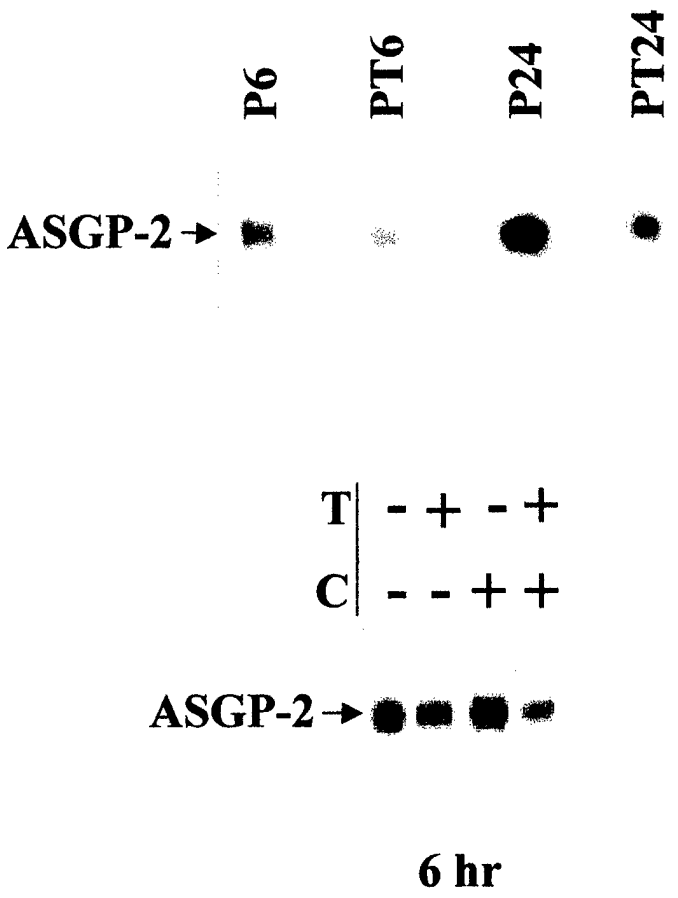
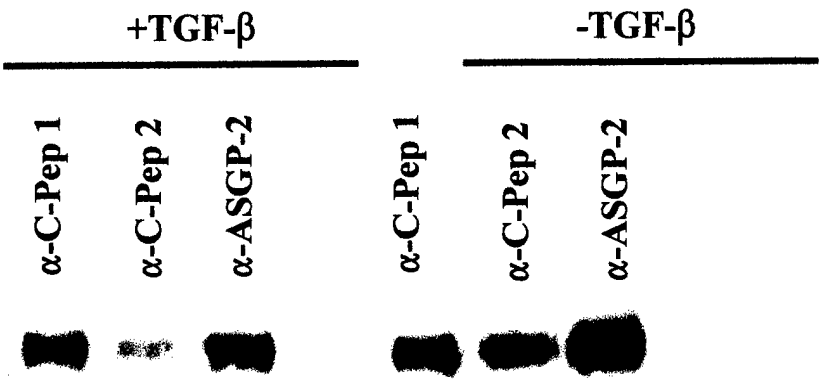


Fig. 4. Timing and effects of cycloheximide on the repression of SMC synthesis by TGFβ. Normal virgin MEC were isolated and cultured on plastic in medium supllemented with 10% fetal calf serum. After 24 hr, the serum-contating medium was replaced and the cells were cultured for an additional 24 hr in the presence or absence of 200 pM TGFβ. (**A**) with or without 10 μg/ml cycloheximide (**B**). Cells were harvested and lysates prepared at 6 and 24 hr as indicated at the top of the figure. 5 μg total protein was subjected to SDS-PAGE and immunoblot analysis with mAb 4F12. P, plastic; PT, plastic + 200 pM TGFβ.

A



B

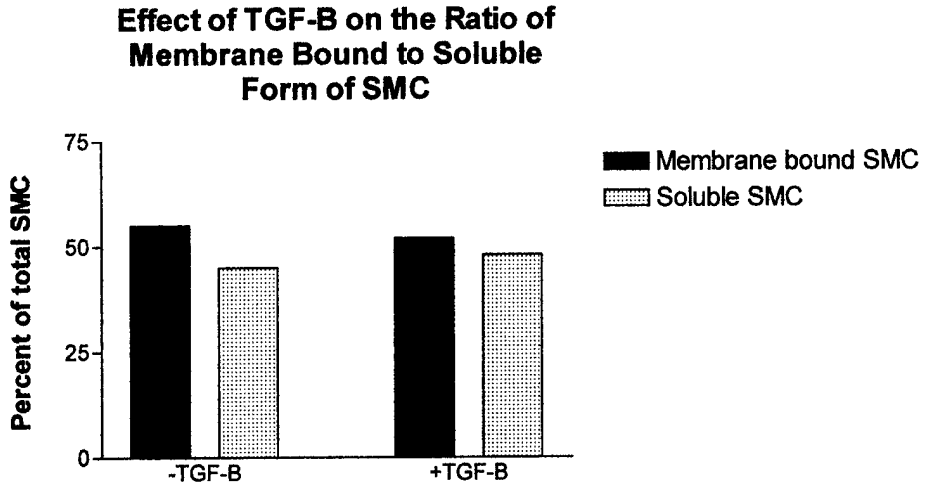
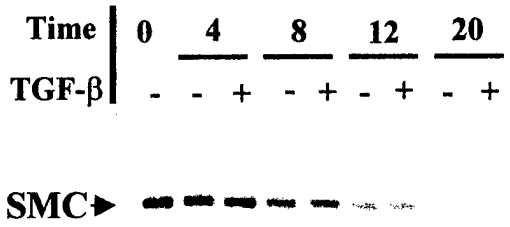


Figure 5. Effect of TGF-β on the ratio of soluble to membrane bound forms of SMC in normal cultured MEC. Virgin MEC were isolated and cultured in the presence or absence of 200 pM TGF-β. After 48 hours cells were harvested and immunoprecipitated twice with anti-C-Pep to clear the lysate of membrane bound form. The lysate was immunoprecipitated with anti-ASGP-2 to recognize the remaining SMC which was not recognized by anti-C-Pep. Immunoprecipitates were subjected to immunoblot analysis with mAb 4F12. A) Western blot of serial immunoprecipitations from MEC cultured in the presence or absence of TGF-β. B) Percentages of membrane bound and soluble forms of SMC in MEC cultured in the presence or absence of TGF-β. The bands from A) were quantified by densitometry and the raw values added for total SMC. Values of both anti-C-Pep bands were added and divided by the total value to get the membrane bound percent. The value of the anti-ASGP-2 band was divided by the total value to get the soluble form percentage, and the results were plotted.

A



B

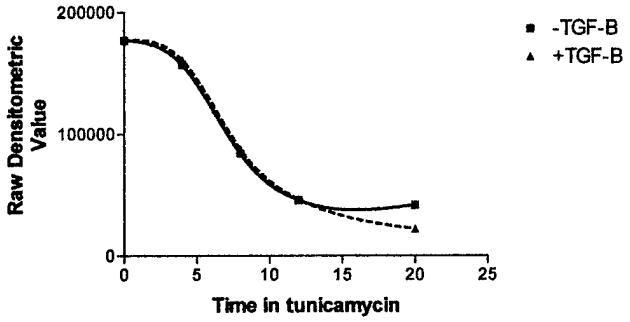
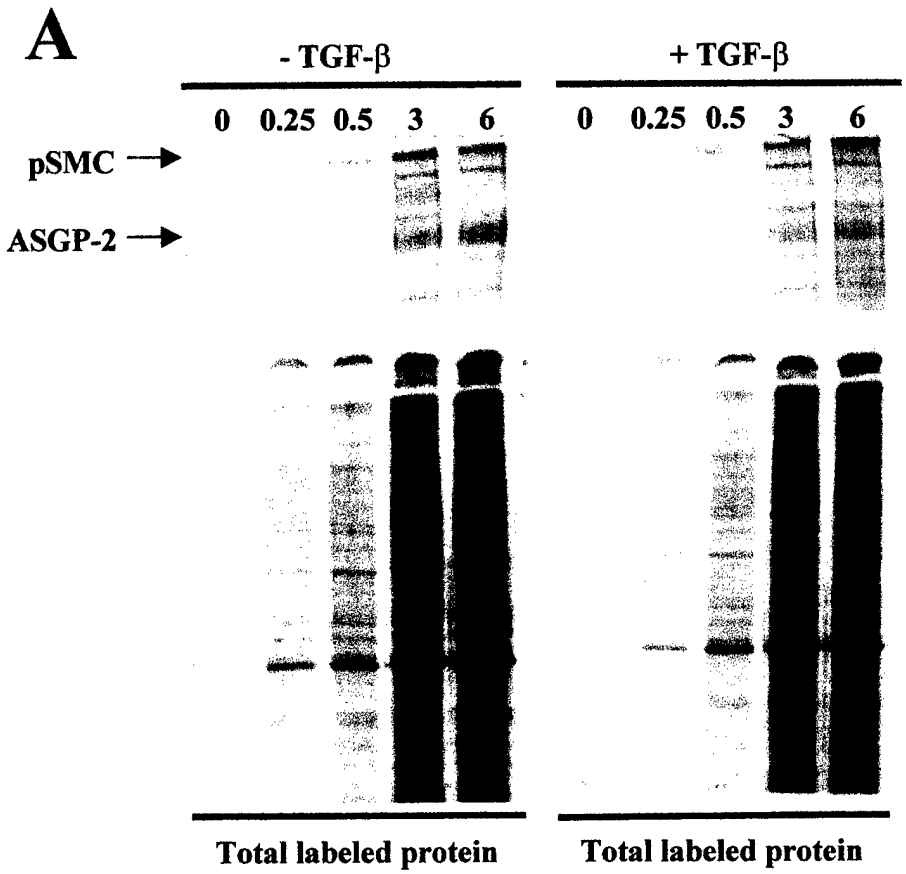


Fig. 6. Effect of TGF-β on the turnover of SMC in normal MEC using tunicamycin as an inhibitor of new SMC synthesis. Virgin MEC were isolated and cultured in the absence of TGF-β. After 24 hours tunicamycin was (5 μg/ml final concentration) was added to all the samples to inhibit new SMC synthesis, and TGF-β (200 pM final) was added to half as indicated at the top of the figure. Samples were taken at various times as indicated at the top of the figure. 5 μg total protein were analyzed by immunoblot with mAb 4F12. A) Western blot of MEC cultured with tunicamycin in the presence or absence of TGF-β. B) Plot of SMC turnover in tunicamycin treated MEC cultured in the presence or absence of TGF-β. The bands from A) were quantified by densitometry, and the results were plotted.



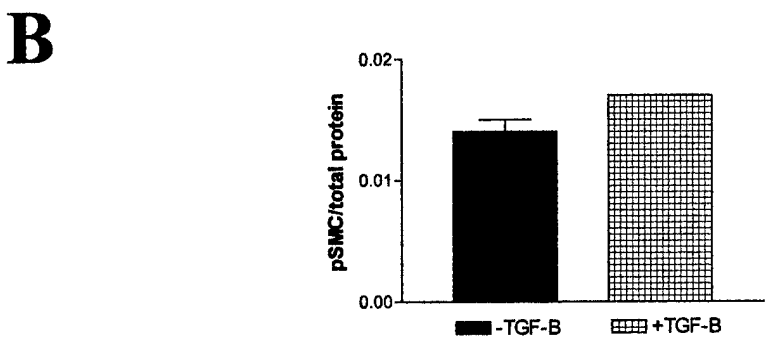


Figure 7. Effect of TGFβ on SMC biosynthesis in normal cultured MEC. A) Virgin MEC were isolated and cultured in the absence of TGFβ . After 24 hours TGFβ (200 pM final concentration) was added to half the samples. After 24 hours cells were metabolically labeled with [35S]-Cys + [35S]-Met for various times as indicated at the top of the figure. Cells were harvested and immunoprecipitated with anti-ASGP-2 polyclonal antibody. Immuoprecipitates and non-immunoprecipitated whole cell lysates were subjected to SDS-PAGE and fluorography. B) Plot of SMC precursor accumulation. The precursor bands from A) were quantified by densitometry. These values were divided by the densitometric values obtained for total labeled protein, and the results were plotted.

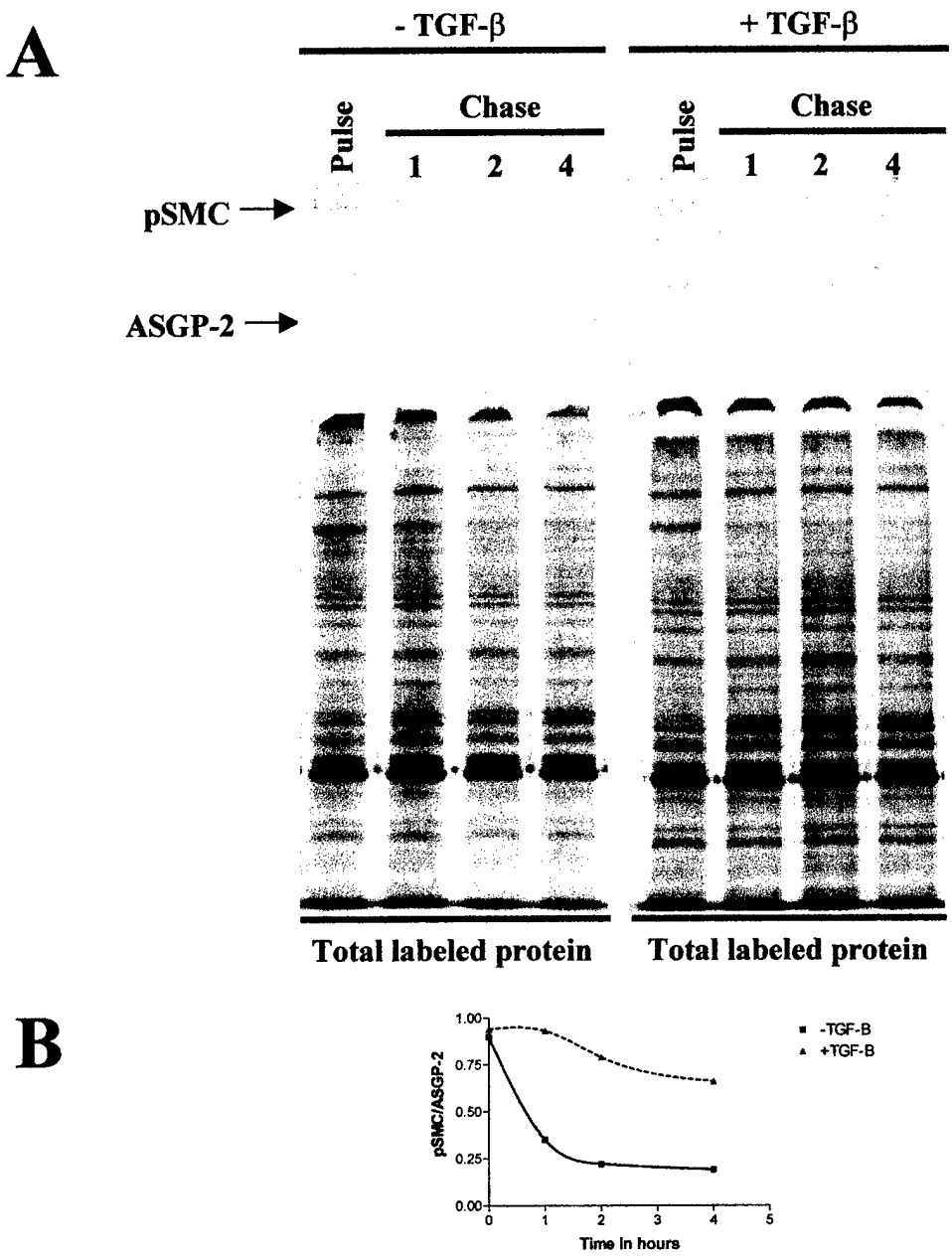


Figure 8. Effect of TGFβ on SMC precursor processing in normal cultured MEC. A) Normal virgin MEC were isolated and cultured in the presence or absence of 200 pM TGFβ. After 48 hours cells were metabolically labeled with [35S]-Cys + [35S]-Met. After a 30 min pulse labeling, the medium was replaced with non-radioactive medium, and cells were harvested at various times as indicated at the top of the figure. Samples were immunoprecipitated, and immunoprecipitates and non-immunoprecipitated whole cell lysate samples were subjected to SDS-PAGE and fluorography. B) Plot of SMC precursor processing into ASGP-2. The precursor and ASGP-2 bands from A) were quantified by densitometry. The value of the precursor band was divided by that for the mature ASGP-2, and the results were plotted.

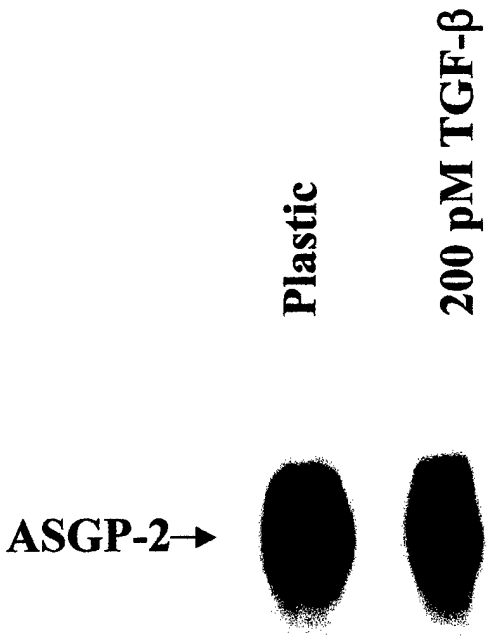


Fig. 9. Effect of TGFβ on SMC levels in cultured 13762 MAT-B1 tumor cells. MAT-B1 tumor cells were collected and cultured on plastic dishes in DMEM medium supplemented with 10% fetal calf serum in the presence or absence of 200 pM TGFβ. After 24 hours, cells were harvested and analyzed by immunoblot with mAb 4F12.

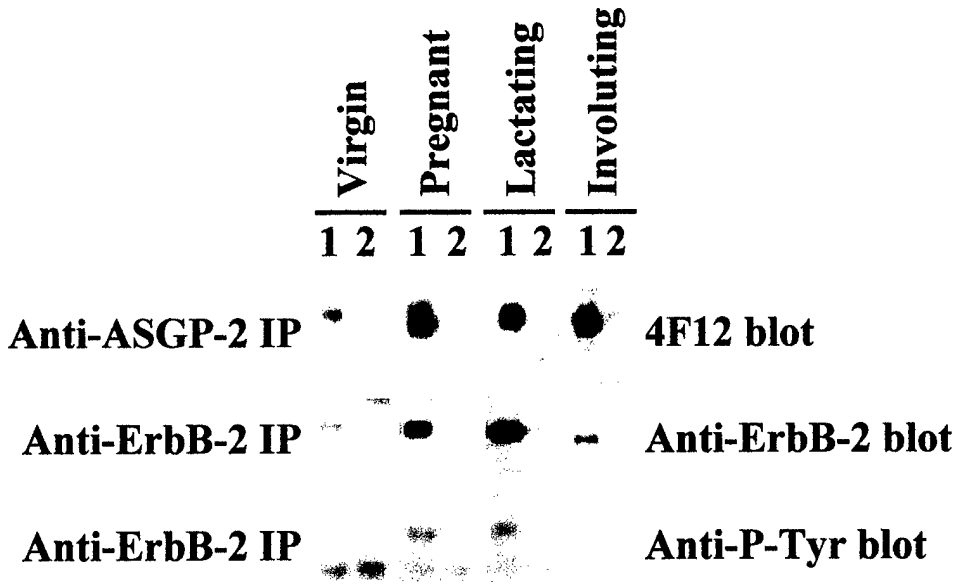


Fig. 10. Time course of SMC and ErbB-2 in normal developing mammary gland. Mammary tissue lysates from virgin, mid-pregnant, lactating, and 2 day involuting rats were immunoprecipitated with anti-ASGP-2, anti-ErbB-2 as indicated at the left of the figure. Immunoprecipitated proteins were subjected to immunoblot with anti-ASGP-2 mAb 4F12, anti-ErbB-2, or anti-phosphotyrosine as indicated at the right of the figure. Lane 1) mammary lysate immunoprecipitated with antibody indicated at left of figure; Lane 2) mammary lysate immunoprecipitated with nonimmune rabbit serum.

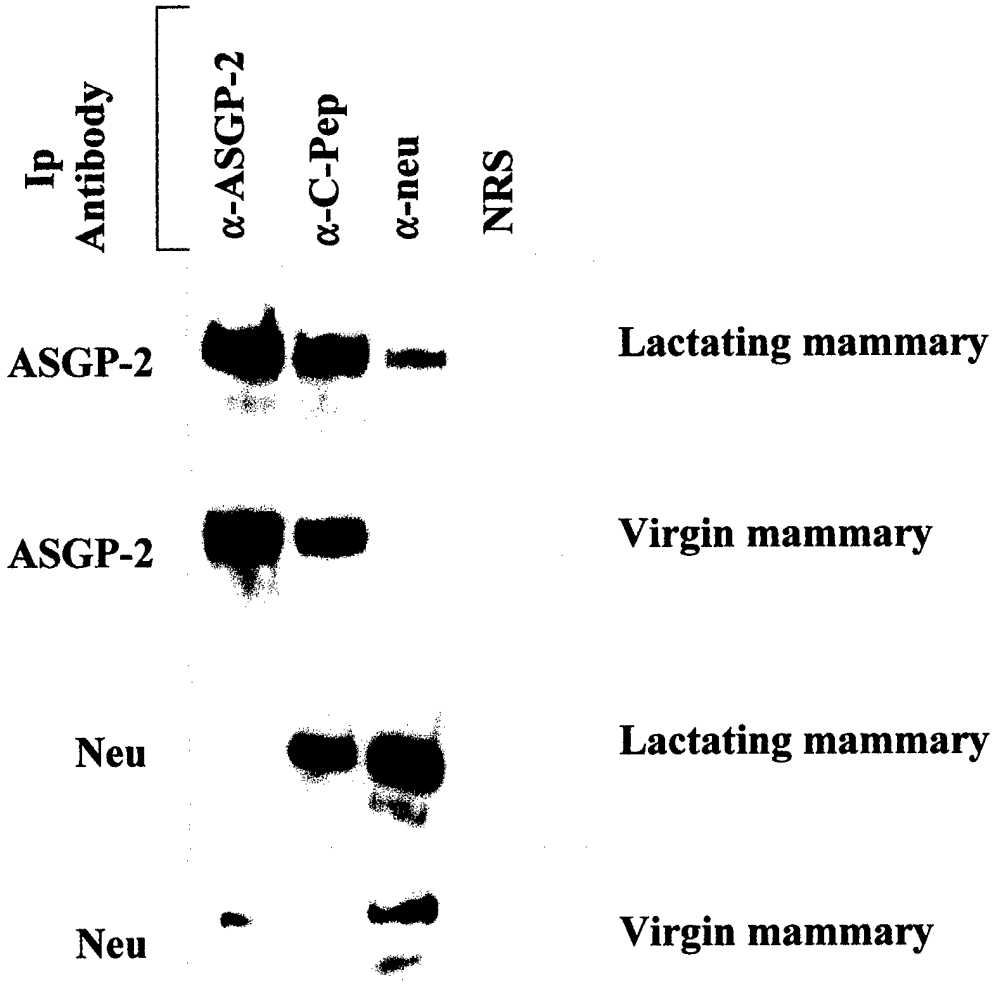


Fig. 11. Co-immunoprecipitation of ASGP-2 and ErbB-2 from virgin and lactating mammary tissue. Mammary tissue lysates from virgin and lactating rats were immunoprecipitated with anti-ASGP-2, anti-C-Pep (anti-ASGP-2 C-terminal antibody), anti-ErbB-2, or nonimmune rabbit serum as indicated at the top of the figure. Immunoprecipitated proteins were subjected to immunoblot with anti-ASGP-2 mAb 4F12 or anti-ErbB-2 antibodies as indicated at the left of the figure.

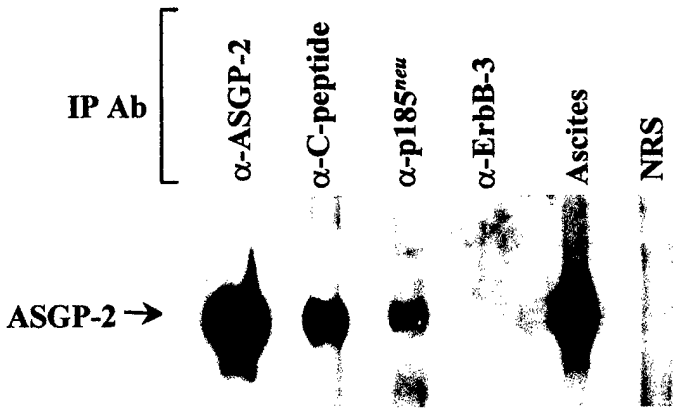


Figure 12. Co-immunoprecipitation of ASGP-2 and ErbB-2, but not ErbB-3, from normal rat mammary epithelial cells. Isolated mammary epithelial cells were solubilized in RIPA buffer and immunoprecipitated with polyclonal antibodies as indicated at the top of the figure. Immunoprecipitates were subjected to immunoblot analysis with a monoclonal antibody against ASGP-2 as indicated to the left of the figure. NRS: non-immune rabbit serum; Ascites: positive control for blot.

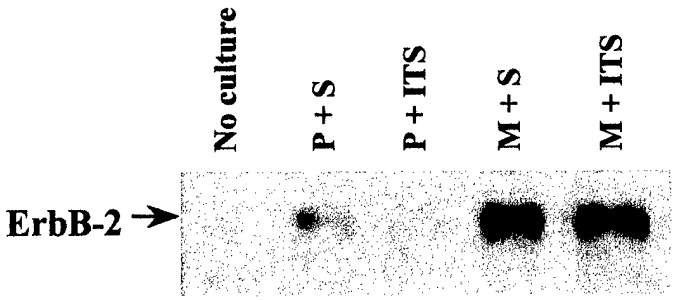


Figure 13. ASGP-2 expression is regulated differently than ErbB-2 in pregnant rat mammary epithelial cells. Normal rat mammary MEC were collected by collagenase digestion of day 11pregnant rat mammary tissue followed by differential centrifugation. MEC were plated in Ham's F-12 media supplemented with lactogenic hormones (prolactin, insulin, and hydrocortisone) either on plastic or embedded in Matrigel in the presence or absence of 10% fetal calf serum. Cells were harvested and washed in PBS prior to SDS-PAGE. Immunoblots were performed with monoclonal antibodies against ErbB-2. P: plastic, M: Matrigel, S: serum, ITS: insulin, transferrin, and selenium. Please refer to accompanying reprint (Price-Schiavi et al., 1998) for ASGP-2 blot for comparison.

References

Carraway, K. L. III, Rossi, E. A., Komatsu, M., Price-Schiavi, S. A., Huang, D., Carvajal, M. E., Guy, P. M., Fregien, N., Carraway, C. A. C., and Carraway, K. L. (1999) *J. Biol. Chem.* **274**, 5263-5266

Carraway, K. L., Fregien, N., Carraway, C. A. C., and Carraway, K. L.III. (1992) *J. Cell Sci.* **103**, 299-307

Elbein, A.D. (1984) *CRC Crit. Rev. Biochem.* **16,** 21-49

Hull, S. R., Sheng, Z., Vanderpuye, O. A., David, C., and Carraway, K. L. (1990) *Biochem. J.* **265,** 121-129

Komatsu, M., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1997) *J. Biol. Chem.* **272,** 33245-33254

Komatsu, M., Yee, L., and Carraway, K.L. (1999) *Cancer Res.* **59,** 2229-2236

McNeer, R.R., Price-Schiavi, S. A., Komatsu, M., Fregien, N., Carraway, C.A.C., and Carraway, K. L. (1997) *Front. Bioscience.* **2,** 449-459

Moniaux, N., Nollet, S., Porchet, N., Degand, P., Laine, A., and Aubert, J.P. (1999) *Biochem. J.* **338,** 325-333

Price-Schiavi, S. A., Carraway, C. A. C., Fregien, N., and Carraway, K. L. (1998) *J. Biol. Chem.* **273**, 35228-35237

Rossi, E. A., McNeer, R., Price-Schiavi, S. A., Komatsu, M., Van den Brande, J. M. H., Thompson, J. F., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1996) *J. Biol. Chem.* **271,** 33476-33485

Sheng, Z., Wu, K., Carraway, K. L., and Fregien, N. (1992) *J. Biol. Chem.* **267,** 16341-16346

Sherblom, A. P., and Carraway, K. L. (1980) *J. Biol. Chem.* **255,** 12051-12059

Sherblom, A. P., Buck, R. L., and Carraway, K. L. (1980) *J. Biol. Chem.* **255,** 783-790

Sherblom, A.P., Huggins, J. W., Chesnut, R.W., Buck, R.L., Ownby, C.L., Dermer, G.B., and Carraway, K.L. (1980) *Exp. Cell Res.* **126**, 417

Wu, K., Fregien, N., and Carraway, K. L. (1994) *J. Biol. Chem.* **269,** 11950-11955

Key research accomplishments to date:

1. SMC is developmentally regulated in normal rat mammary gland largely by a post-transcriptional mechanism. (Discussed in last progress report.)

2. Matrigel (reconstituted ECM) post-transcriptionally regulates SMC levels in normal rat MEC by inhibition of SMC precursor synthesis. (Discussed in last progress report.)

3. SMC levels in 13762 MAT-B1 tumor cells are unaffected by Matrigel. (Discussed in last progress report.)

4. SMC is post-translationally regulated in normal rat MEC by TGFβ by disruption of SMC precursor processing. (Note that this is a different mechanism than that described for Matrigel.)

5. SMC expression is unaffected by TGFβ in 13762 MAT-B1 tumor cells.

6. SMC and ErbB-2 have similar expression patterns in normal developing rat mammary gland.

7. SMC and ErbB-2 can form a complex in both virgin and lactating mammary gland.

8. SMC and ErbB-2 have different mechanisms of regulation in cultured normal rat MEC.

Reportable outcomes:

1. Papers:

   Price-Schiavi, S.A., Carraway, C.A.C., Fregien, N.L., and Carraway, K.L. (1998) Post-transcriptional regulation of a milk membrane protein, the sialomucin complex (ascites sialoglycoprotein (ASGP)-1/ASGP-2, rat Muc4), by transforming growth factor β *J. Biol. Chem.* **273**, 35228-35237

2. Abstracts and presentations:

   Post-transcriptional regulation of a milk membrane protein, Sialomucin complex, by TGFβ
   Price-Schiavi, S.A., Carraway, C.A.C., Fregien, N.L., and Carraway, K.L.
   Poster presentation at American Society for Cell Biology, San Franscisco CA, December, 1998

   Post-transcriptional regulation of sialomucin complex in normal rat mammary gland by TGFβ
   Price-Schiavi, S.A., Fregien, N.L., Carraway, C.A.C., and Carraway, K.L.
   Poster presentation at Nature BioTechnology Winter Symposium, Miami FL, February, 1999

   Characterization of the TGFβ effect on sialomucin complex (Rat MUC-4) expression in normal rat mammary epithelial cells
   Price-Schiavi, S.A., Zhu, X., and Carraway, K.L.
   Poster presentation at American Society for Biochemistry and Molecular Biology, San Francisco CA, May 1999

   Mechanisms for post-transcriptional regulation of SMC expression in normal rat mammary epithelial cells
   Price-Schiavi, S.A., Aquinin, R., and Carraway, K.L.
   Poster presentation at Gordon Conference on Mammary Gland Biology, 1999

Abstract for ASCB 1998

POST-TRANSCRIPTIONAL REGULATION OF A MILK MEMBRANE PROTEIN SIALOMUCIN COMPLEX BY TGF-β. ((Shari A. Price-Schiavi, Coralie A. Carothers Carraway, Nevis Fregien, and Kermit L. Carraway)) Departments of Cell Biology and Anatomy and Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL 33101

Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which is highly overexpressed on the surface of ascites 13762 rat mammary adenocarcinoma cells. In normal rat mammary gland SMC is sharply increased at mid-pregnancy in a manner similar to β-casein. Unlike β-casein, SMC appears to be regulated posttranscriptionally, as its transcript is present in both virgin and pregnant mammary tissue and SMC synthesis is induced rapidly in cultured primary mammary epithelial cells from either normal pregnant or virgin rats. SMC protein, but not transcript levels, are significantly reduced when mammary cells are cultured in Matrigel, a reconstituted basement membrane which stimulates casein expression. SMC precursor is synthesized in Matrigel at a 10-fold lower rate than when mammary cells are cultured on plastic. Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. The Matrigel effect on primary mammary and 13762 cells is mimicked by TGF-β, a component of this complex matrix. These results indicate that SMC is a novel product of normal mammary gland and milk, which is post-transcriptionally regulated by TGF-β in normal mammary gland, but not in 13762 mammary adenocarcinoma cells.

Abstract report for Nature BioTechnology Winter Symposium 1999

POST-TRANSCRIPTIONAL REGULATION OF SIALOMUCIN COMPLEX IN NORMAL RAT MAMMARY GLAND BY TGF-β

Shari A. Price-Schiavi, Nevis L. Fregien, Coralie A. C. Carraway, Kermit L. Carraway. Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, FL 33136

INTRODUCTION Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 and a transmembrane subunit ASGP-2, which is highly overexpressed on the surface of ascites 13762 rat mammary adenocarcinoma cells.[1] SMC is also expressed in a number of normal rat tissues including small and large intestine, trachea, uterus, and mammary gland.[2-4] SMC is expressed constitutively in the trachea and small and large intestine, while in the uterus its expression is under the control of estrogen and progesterone. The wide tissue distribution and expression pattern suggests that SMC has multiple and complex regulatory mechanisms.

METHODS Normal rat mammary epithelial cell cultures were established by collagenase digestion of normal virgin rat mammary tissue. Isolated epithelial cells were cultured on plastic or embedded in Matrigel in Ham's F-12 medium supplemented with 5 ug/ml insulin, transferrin, sodium selenite and various concentrations of TGF-β. After 24 to 48 hours cells were harvested and the resulting lysates (5 ug/lane) were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. In addition, total RNA was prepared and subjected to Northern blot analysis with a probe spanning the 5' unique region of SMC cDNA.

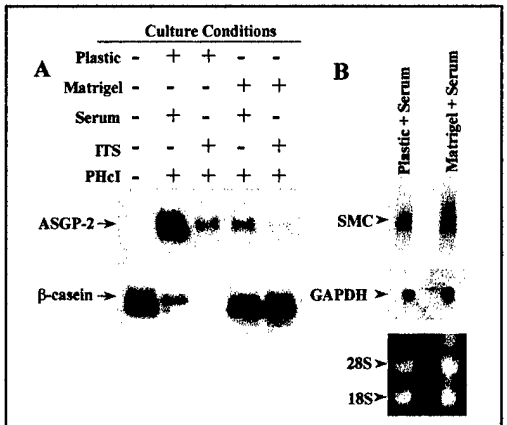


Figure 1

RESULTS In cultured normal rat mammary epithelial cells, SMC protein, but not transcript, is significantly reduced when the cells are cultured in Matrigel, a reconstituted basement membrane (Fig. 1). SMC precursor in synthesized in Matrigel at a 10-fold lower rate than when mammary cells are cultured on plastic, suggesting that regulation of SMC occurs at the level of translation under these conditions. The Matrigel effect on primary mammary and 13762 cells can be mimicked by TGF-β, a component of this complex matrix (Fig. 2), suggesting that TGF-β may be the factor responsible for post-transcriptional regulation of SMC.

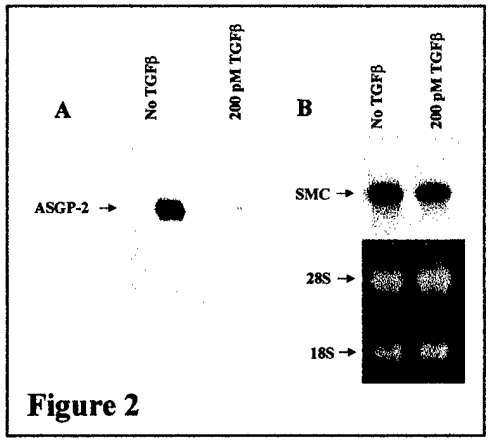


Figure 2

CONCLUSION These results indicate that SMC is a novel product of normal mammary gland and milk, which is post-transcriptionally regulated by TGF-β in normal mammary gland, but not in 13762 mammary adenocarcinoma cells.

REFERENCES

1. Sherblom, A. P., and Carraway, K. L. (1980) *J. Biol. Chem.* **255,** 12051-12059.
2. Rossi, E. A., McNeer, R., Price-Schiavi, S. A., Komatsu, M., Van den Brande, J. M. H., Thompson, J. F., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1996) *J. Biol. Chem.* **271,** 33476-33485.
3. McNeer, R. R., Carraway, C. A. C., Fregien, N., and Carraway, K. L. (1998) *J. Cell. Physiol.* **176,** 110-119.
4. McNeer, R. R., Huang, D., Fregien, N., and K. L. Carraway (1998) *Biochem. J.* **330,** 737-744.

Abstract for ASBMB 1999

Characterization of the TGF-β effect on sialomucin complex (Rat MUC-4) expression in normal rat mammary epithelial cells ((Shari A. Price-Schiavi, Xiaoyun Zhu, and Kermit L. Carraway)) *Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami FL 33101*

Sialomucin complex (SMC) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which is highly overexpressed on the surface of ascites 13762 rat mammary adenocarcinoma cells. In normal rat mammary gland SMC, which is present in both membrane bound and soluble forms, is sharply increased at mid-pregnancy and is regulated post-transcriptionally by TGF-β. The presence of TGF-β significantly decreases the level of SMC protein but not the SMC transcript. Interestingly, SMC expression in the 13762 mammary adenocarcinoma cells is unaffected by TGF-β. Reduction of SMC levels in normal mammary epithelial cells by TGF-β is reversible by addition of a neutralizing TGF-β antibody or by removal to TGF-β from the culture medium. The reduction is fast (less than or equal to 6 hours) and occurs several hours before TGF-β induced cell cycle arrest, indicating that this effect is independent of cell cycle effects. The presence of TGF-β does not alter the ratio of membrane bound to soluble forms of SMC, suggesting that the apparent reduction of SMC levels in these cells is not due to an increase in the synthesis of the soluble form. The addition of cycloheximide to the culture medium does not change the effect of TGF-β on SMC levels, indicating that no new protein synthesis is necessary for this effect. These results suggest that TGF-β regulates SMC expression by an effect on its translation.

Poster presentation for Gordon Conference on Mammary Gland Biology 1999

Mechanisms for post-transcriptional regulation of SMC expression in normal rat mammary epithelial cells

Shari A. Price-Schiavi, Ronny Aquinin, and Kermit L. Carraway
Department of Cell Biology and Anatomy, University of Miami School of Medicine Miami FL 33136

Sialomucin complex (SMC, rat Muc4) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, produced from a single gene and precursor. SMC expression is tightly regulated in mammary gland; the level in lactating mammary gland is about 100-fold that in virgin gland. In rat mammary epithelial cells, SMC is post-transcriptionally regulated by Matrigel by inhibition of SMC precursor synthesis. SMC is also post-transcriptionally regulated by transforming growth factor-β (TGFβ). The repression of SMC expression by TGFβ is rapid, is independent of TGFβ-induced cell cycle arrest and does not require new protein synthesis. Unlike Matrigel, TGFβ does not reduce SMC protein synthesis, as SMC precursor accumulation is equivalent in TGFβ-treated and untreated cells. Instead, SMC precursor in TGFβ treated cells is more persistent and does not become processed as rapidly into mature ASGP-1 and ASGP-2, indicating that TGFβ disrupts processing of SMC precursor. These results indicate that SMC, a product of normal mammary gland and milk, is regulated by TGFβ by a novel post-translational mechanism. Thus, SMC is regulated by multiple post-transcriptional mechanisms, which serve to repress potential deleterious effects of overexpression.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 52, Issue of December 25, pp. 35228–35237, 1998
Printed in U.S.A.

Post-transcriptional Regulation of a Milk Membrane Protein, the Sialomucin Complex (Ascites Sialoglycoprotein (ASGP)-1/ASGP-2, Rat Muc4), by Transforming Growth Factor β*

(Received for publication, August 13, 1998, and in revised form, October 19, 1998)

Shari A. Price-Schiavi‡, Coralie A. Carothers Carraway§, Nevis Fregien‡, and Kermit L. Carraway‡¶

From the Departments of ‡Cell Biology and Anatomy and §Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida 33101

Sialomucin complex (SMC, Rat Muc4) is a heterodimeric glycoprotein complex consisting of a mucin subunit ASGP-1 (ascites sialoglycoprotein-1) and a transmembrane subunit ASGP-2, which can act as a ligand for the receptor tyrosine kinase ErbB2. SMC is highly expressed on the surface of ascites 13762 rat mammary adenocarcinoma cells, approximately 100 times the level in lactating mammary gland and 10^4 times that in virgin mammary gland. SMC is sharply increased at mid-pregnancy in a manner similar to β-casein. Unlike β-casein, SMC appears to be regulated post-transcriptionally. Its transcript is present in both virgin and pregnant mammary tissue, and SMC synthesis is induced rapidly in cultured primary mammary epithelial cells from either normal pregnant or virgin rats. SMC protein, but not transcript, levels are significantly reduced when mammary cells are cultured in Matrigel, a reconstituted basement membrane which stimulates casein expression. SMC precursor is synthesized in Matrigel at a 10-fold lower rate. Matrigel has no effect on either the level of SMC or its transcript in cultured 13762 mammary tumor cells. The Matrigel effect on primary mammary and 13762 cells is mimicked by transforming growth factor β, a component associated with this complex matrix. These results indicate that SMC is a novel product of normal mammary gland and milk, which is post-transcriptionally regulated by transforming growth factor β in normal mammary gland, but not in 13762 mammary adenocarcinoma cells.

Mammary gland development and differentiation are regulated by complex interactions of growth factors, hormones, and ECM[1] (1–5). The regulation of some mammary specific milk proteins by ECM has been studied in detail. For example, β-casein, an "early" milk protein which is detectable around day 6 of pregnancy in the mouse, is transcriptionally regulated by laminin through β1 integrins (6, 7). Whey acidic protein (WAP), a "late" milk protein whose message becomes detectable around day 14 of pregnancy in the mouse, is dependent on an ECM-induced three-dimensional alveolar structure (6). When normal mammary epithelial cells (MEC) are placed in culture, WAP transcription is unaffected by the presence or absence of basement membrane, suggesting an additional post-transcriptional regulatory mechanism (6). Thus, both the combinations of factors necessary for proper regulation of expression of milk protein genes and the mechanisms of regulation are complex.

SMC, which was recently identified as one of the milk membrane mucins (8), was originally discovered as the major glycoprotein complex on the surface of highly malignant, metastatic 13762 rat ascites mammary adenocarcinoma cells (9, 10). The complex consists of an *O*-glycosylated mucin subunit ASGP-1 (9–12), which is tightly, but non-covalently, bound to an *N*-glycosylated integral membrane glycoprotein ASGP-2 (10, 13). SMC is transcribed from a single gene as a 9-kilobase transcript (14, 15), which is translated into a single polypeptide which is proteolytically cleaved early in its transit to the cell surface (16). Mature glycosylated ASGP-1 has a molecular mass of >500 kDa (9), with a polypeptide molecular mass of ≈220 kDa (15), and comprises three domains: an N-terminal unique sequence, a large tandem repeat region rich in serine and threonine residues similar to that of other mucins, and a C-terminal unique sequence (15). ASGP-2 is a 120–140-kDa protein that consists of seven domains: two hydrophilic *N*-glycosylated regions, two EGF-like domains, a cysteine-rich domain, a transmembrane domain, and a small cytoplasmic domain (14).

In tumor cells sialomucin complex may have dual functions. 1) ASGP-1 can provide anti-recognition and anti-adhesive properties to tumor cells (11, 12, 18). In A375 melanoma cells stably transfected with SMC DNA linked to a tetracycline-regulated promoter, expression of SMC abolishes cell-matrix adhesion and cell-cell interactions (18). Furthermore, SMC expression in these cells reduces their killing by natural killer cells.[2] This anti-recognition property may be important to the high metastatic capacity of the 13762 ascites cells (9, 11, 19). 2) The two EGF-like domains have all of the consensus residues present in active members of the EGF growth factor family (14), and SMC has been shown to bind to the receptor ErbB2. Thus, the transmembrane subunit ASGP-2 is proposed to modulate signaling through the EGF family of receptors via its interaction with ErbB2,[3] the critical receptor for formation of

* This work was supported in part by Grant CA 52498 from the National Institutes of Health, and Sylvester Comprehensive Cancer Center, National Institutes of Health Grant CA 14395. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Cell Biology (R-124), University of Miami School of Medicine, P. O. Box 016960, Miami, FL 33101. E-mail: kcarrawa@mednet.med.miami.edu.

[1] The abbreviations used are: ECM, extracellular matrix; WAP, whey acidic protein; SMC, sialomucin complex; ASGP, ascites sialoglycoprotein; EGF, epidermal growth factor; MEC, mammary epithelial cells; PBS, Dulbecco's phosphate-buffered saline without calcium; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; TGFβ, transforming growth factor β.

[2] M. Komatsu, C. A. C. Carraway, N. Fregien, and K. L. Carraway, manuscript in preparation.

[3] K. L. Carraway III, E. A. Rossi, S. A. Price-Schiavi, M. Komatsu, D. Huang, P. M. Guy, M. E. Carvajal, N. Fregien, C. A. C. Carraway, and K. L. Carraway, submitted for publication.

active heterodimeric class I receptor tyrosine kinases (20), for which no ligand has been described to date. This interaction may play a role in the constitutive phosphorylation of ErbB2 in the 13762 ascites cells (21) and the high proliferative activity of these cells.

Sialomucin complex is not mammary specific. It is expressed in a number of normal secretory epithelial tissues in the adult rat, including small and large intestine, trachea, and uterus (8, 17). In the trachea it is constitutively expressed on the apical surface of the cells lining the lumen (17). SMC is also constitutively expressed in the normal adult rat intestine. In the adult small intestine SMC is observed only in Paneth cells at the bases of the crypts of Lieberkuhn, while in the colon it is found specifically in goblet cells (8). In the uterus, SMC is observed on the luminal surface of the endometrium and in glandular cells. Its expression is regulated at the mRNA level by the ovarian hormones estrogen and progesterone (22). These results suggest that SMC has multiple and complex regulatory mechanisms in the normal adult rat.

SMC is abundant in milk (ASGP-2 concentration, ~10 μg/ml) and lactating mammary gland, but its level is very low in the virgin gland. In lactating mammary tissue it is localized at the apical surface of secretory epithelial cells lining the alveoli. It appears to be located within the cytoplasm, not limited to the plasma membrane or milk fat globule membrane, suggesting that its location is in the mammary secretory granules (8). In this tissue SMC is expressed as both membrane and soluble forms, the soluble form lacking the transmembrane and C-terminal domains. Interestingly, the level of SMC in the 13762 ascites cells is about 100-fold greater than that in the lactating mammary gland (8). These results suggest that SMC is developmentally regulated in normal mammary gland, but that this regulation is lost in the rat mammary ascites tumor.

Recent studies have helped to define the place of SMC among the hierarchy of known mucins. Cloning and sequencing of full-length human MUC4 show substantial similarities between the MUC4 and rat SMC at the N– and C-terminal portions of the molecules (23).[4] They differ in their repeat domains. In particular, SMC does not have the 16-amino acid repeat cloned and sequenced in the original description of MUC4 (24). Thus, the similarity between the molecules was not observed previously. However, the 70% identity between the human MUC4 analog of ASGP-2 and rat ASGP-2 provides strong evidence that they are homologous proteins.

In an effort to understand the regulation of SMC in normal mammary gland, we have examined both mammary tissue and cultures of primary mammary epithelial cells. *In vivo* SMC expression is superficially similar to that of β-casein and is clearly developmentally regulated. *In vitro*, however, in contrast to β-casein, which requires ECM for expression, SMC levels are significantly reduced in the presence of Matrigel, a reconstituted ECM preparation. Unlike β-casein, high levels of SMC can be induced within hours in cultured virgin mammary epithelial cells. Furthermore, SMC message is expressed at all times in the normal developing mammary gland, unlike the protein, which is not present in virgin gland and increases as pregnancy proceeds. In addition, TGFβ can reduce the level of SMC protein without affecting the level of SMC transcript, suggesting that it is the factor responsible for post-transcriptional regulation of SMC in normal MEC. These data suggest that SMC is a novel post-transcriptionally regulated product of the mammary gland and milk.

[4] J.-P. Aubert, results presented at the Fifth Workshop on Tumor Mucins, Cambridge, United Kingdom.

EXPERIMENTAL PROCEDURES

Materials—The MAT-B1 ascites subline of the 13762 rat mammary adenocarcinoma was maintained by weekly passage, as described previously (25). Anti-ASGP-2 polyclonal antiserum used for immunoprecipitations (16) and mouse monoclonal antibody 4F12 used for immunoblots (8) have been described previously. Anti-β-casein mouse monoclonal antibody used for immunoblots was kindly provided by Dr. Charlotte Kaetzel (University of Kentucky, Lexington, KY). TGFβ was purchased from R & D Systems, Inc. Cell culture materials were obtained from Life Technologies, Inc., unless otherwise noted.

Blotting Procedures—For Northern blots total RNA was isolated from whole normal mammary tissue, mammary epithelial cells, or 13762 MAT-B1 cells using TRI REAGENT™ (Molecular Research Center, Inc., Cincinnati, OH), and 25 μg were electrophoresed on 1% formaldehyde/agarose gels. Resolved RNAs were transferred to Zeta-Probe positively charged nylon membranes (Bio-Rad), followed by cross-linking using a Stratalinker (Stratagene, La Jolla, CA). The membranes were prehybridized for at least 2 h at 42 °C in prehybridization solution (50% formamide, 5 × SSC, 5 × Denhardt's reagent, 0.1% SDS, and 0.5 mg/ml salmon sperm DNA). The probe, A2G2--9, a 1.7-kilobase probe which spans the 5′ unique region and four tandem repeats of SMC cDNA, was random primed labeled with [^{32}P]dCTP using a Random Primed Labeling kit (Boehringer-Mannheim). The membranes were hybridized overnight at 42 °C in prehybridization solution containing 0.1 g/ml dextran sulfate and the labeled probe. Following hybridization, membranes were washed once at room temperature in 2 × SSC with 0.1% SDS for 15 min, twice at 50 °C in 2 × SSC with 0.1% SDS for 20 min each, and once at 50 °C in 0.1X SSC with 0.1% SDS for 15 min. Signals were detected by exposure with Kodak XAR-5 x-ray film.

For Western blots, SDS-PAGE was performed under reducing conditions using 6% polyacrylamide gels and the mini-Protean II system (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes which were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline with 0.5% Tween 20. After a 1-h incubation in primary antibody diluted in 1% bovine serum albumin/Tris-buffered saline with 0.5% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG Fc-specific (Pierce, Rockford, IL) diluted 1:20,000 in 1% bovine serum albumin/Tris-buffered saline with 0.5% Tween 20. Signals were detected with the Renaissance™ Enhanced Chemiluminescence kit (NEN Life Science Products Inc., Boston, MA).

Preparation of Tissue Samples—Mammary tissue dissected from female Fischer 344 rats was pulverized with a mortar and pestle in liquid N_2 and stored as a powder at −80 °C. For immunoblotting, tissue powders were solubilized directly into SDS-PAGE sample buffer at a concentration of 10 mg/ml wet weight. For quantitation of total protein, powders were solubilized in 1% SDS in water, boiled, and clarified by centrifugation at 12,000 × *g*. Protein concentrations were determined by Lowry assay of the cleared SDS lysates.

Preparation of Mammary Epithelial Cells—Primary mammary epithelial cell cultures were established using previously described protocols (26–29). Briefly, mammary glands excised from virgin or pregnant female Fischer 344 rats were minced, resuspended in digestion media comprised of 1 mg/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ), and 100 units/ml penicillin, 100 μg/ml streptomycin in Ham's F-12 medium (Life Technologies, Inc.) and incubated at 37 °C with shaking for 45 min. Fully and partially digested epithelial cell clusters were pelleted and incubated a second time in digestion buffer at 37 °C with shaking for 45 min. Digested epithelial cell clusters were pelleted, resuspended in PBS, and passed through a 520-μm cell sieve to remove undigested material. Mammary epithelial cell clusters in the resulting filtrate were captured on a 70-μm nylon membrane. Cell clusters were collected by rinsing the membrane with PBS and were subsequently washed three times in PBS prior to plating. Incubating freshly isolated cells on a plastic tissue culture plate for 1 h permitted attachment and removal of fibroblasts.

Cell Culture and Analysis—Mammary epithelial cell clusters were resuspended and plated in equal aliquots in Ham's F-12 medium containing 1 mg/ml bovine serum albumin (Sigma) and either 10% FCS and 100 units/ml penicillin, 100 μg/ml streptomycin or 5 μg/ml insulin, 10 μg/ml transferrin, 0.3 μg/ml sodium selenite, and 100 units/ml penicillin, 100 μg/ml streptomycin. Where indicated, media were supplemented with 5 μg/ml insulin, 1 μg/ml hydrocortisone, 3 μg/ml prolactin, and/or various concentrations (2.5–20 ng/ml) of TGFβ. For plating with embedding in ECM (Matrigel, Collaborative Biomedical Products, Bedford MA), cells were resuspended in 1.5 ml of ice-cold Matrigel diluted

1:3 with serum-free Ham's F-12 medium, plated at 1.5 μl/mm^2 of tissue culture plastic, and allowed to solidify at 37 °C for 30 min. The solidified Matrigel was then overlaid with 2 ml of either serum-free or serum-containing medium. Cells were cultured at 37 °C in 5% CO_2 for 48 h prior to harvest. Cells were collected from culture on plastic dishes by scraping cells off the dish. Cells were harvested from Matrigel cultures using the recommended protocol for Matrisperse (Collaborative Biomedical Products, Bedford, MA), an enzyme-free Matrigel dissociation buffer. For all samples, harvested cells were pelleted, washed with PBS, and lysed in 100 μl of 1% SDS in water. Protein concentration of the cell lysates was determined by Lowry assay, and 5 μg of total protein was loaded for immunoblot analysis.

Labeling of Mammary Epithelial Cells—Mammary epithelial cells were isolated from virgin rats and cultured either on plastic or embedded in Matrigel in serum-free medium. After 24 h cells were washed twice with PBS, starved for 30 min in Cys/Met free Dulbecco's minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10 mM Hepes, and incubated in 1 ml of labeling medium (starvation medium + 230 μCi of [^{35}S]Cys + [^{35}S]Met per ml) (EXPRE^{35}S^{35}S Protein Labeling Mix, NEN Life Science Products) for times ranging from 5 to 180 min. Labeled cells were washed twice with PBS and lysed in 400 μl of 2% SDS in H_2O. Lysed cells were boiled for 1 min, sonicated for 10 min in a bath sonicator, and diluted in 2 ml of Triton immunoprecipitation buffer (2.5% Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, 6 mM EDTA, pH 7.4). Diluted cell lysates were centrifuged at 175,000 × *g* for 40 min at 4 °C. Cell lysates were immunoprecipitated with polyclonal anti-ASGP-2 antiserum and protein A-agarose beads (Sigma) overnight at 4 °C with rotation. Immunoprecipitates were washed with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris base, pH 8.0) six times for 15 min each at 4 °C with rotation. A fraction of immunoprecipitation supernatant was collected for analysis of total labeled protein. Washed immunoprecipitates were resuspended in 20 μl of SDS sample buffer and immunoprecipitate supernatant was diluted 1:1 in SDS sample buffer. Diluted samples were analyzed by SDS-PAGE and fluorography with Fluoro-Hance autoradiography enhancer (Research Products International Corp., Mount Prospect, IL).

RESULTS

Time Course of SMC Expression in Normal Developing Mammary Gland—Mammary homogenates from virgin, pregnant, lactating, and involuting rats were analyzed by anti-ASGP-2 and anti-β-casein immunoblots to study the expression pattern of SMC in normal developing mammary gland and to compare its expression pattern to that of β-casein. In all tissues studied to date, including mammary gland (8, 17), ASGP-1 and ASGP-2 are present as a complex, allowing us to use immunoblotting of ASGP-2 for the analysis of SMC. Moreover, our antibodies to ASGP-2 are more sensitive and more specific than those for ASGP-1. As previously reported (8), SMC is minimal in virgin mammary gland. Its level increases sharply (approximately 40-fold) during mid-pregnancy, reaches a maximum in late pregnancy and during lactation, and decreases during involution (Figs. 1, *A* and *B*). The expression pattern of β-casein is similar, but it reaches maximal levels about 1 to 2 days earlier than SMC. β-Casein is maintained throughout lactation and decreases more rapidly than SMC during involution. These data suggest that, like β-casein, SMC is developmentally regulated in normal mammary gland, but that it may have a different regulatory mechanism.

The mammary gland is made up of several cell types. SMC is expressed in the secretory luminal epithelial cells (8). Thus, it is necessary to study the level of SMC in isolated MEC to determine if the apparent increase in SMC in developing mammary gland is a consequence of a real increase in SMC production or an increase in the proportion of epithelial cells relative to other cell types during pregnancy. For this reason mammary epithelial cells were isolated by collagenase digestion from virgin and pregnant rats, and anti-ASGP-2 and anti-β-casein immunoblots were performed. To test whether cell surface SMC is degraded by collagenase, MAT-B1 ascites cells were treated with collagenase under the same conditions. Immunoblots of collagenase-treated ascites cells showed no difference in the level of SMC compared with that of untreated cells (data not shown). As seen in whole tissue, SMC is low in virgin mammary epithelial cells and increases sharply in MEC from mid- and late-pregnant animals (Fig. 1*C*). β-Casein expression is also minimal in virgin MEC and is detectable at maximum levels in MEC from both mid- and late-pregnant animals. As seen in whole tissue, β-casein reaches maximum levels earlier than SMC. Thus, the expression of these proteins in the isolated cells reflects that in the mammary tissue and thus can be used as a model for the study of regulatory mechanisms.

To determine whether the expression pattern of the SMC transcript is similar to that for the protein, total RNA was prepared from mammary epithelial cells freshly isolated from virgin and pregnant rats, and Northern blot analysis was performed using probe A2G2-9, which was generated from the 5′ 1.7-kilobases of SMC cDNA. Surprisingly, SMC transcript is present at equivalent levels in MEC at all time points tested (Fig. 2). This expression pattern for SMC transcript is different from that for the protein, which is low in virgin gland and increases sharply as pregnancy proceeds (Fig. 1). Furthermore, this pattern also differs from that of β-casein message, which is undetectable in MEC until mid-pregnancy (6). These results suggest that, unlike β-casein, regulation of SMC in normal rat mammary epithelial cells *in vivo* occurs post-transcriptionally.

Expression of SMC Is Regulated by Matrigel—Primary culture of mammary epithelial cells has been used to elucidate the regulatory mechanisms of several milk proteins, including β-casein. These systems are useful because the level of differentiation/functionality can be maintained and manipulated. In the presence of ECM and lactogenic hormones (insulin, hydrocortisone, and prolactin), isolated mammary epithelial cells will form round alveolar structures with closed lumens and will vectorially secrete milk proteins. On plastic these cells form squamous epithelial monolayers and are considered undifferentiated because milk proteins are not synthesized. The *in vivo* expression patterns of SMC and β-casein are different, suggesting that SMC may be regulated differently than β-casein or other early milk protein genes. Expression of β-casein in isolated mammary epithelial cells is dependent on the presence of extracellular matrix components (or basement membrane). In the presence of a reconstituted basement membrane (Matrigel), mammary epithelial cells from pregnant mice and virgin rats can be induced to express β-casein. To investigate the effect of extracellular matrix on SMC, mammary epithelial cells were isolated from mid-pregnant (day 11) rats, when SMC is low and casein is high. Cells were cultured either on plastic or embedded in Matrigel in the presence or absence of FCS. After 48 h, cleared cell lysates were prepared for anti-ASGP-2 and anti-β-casein immunoblots, and total RNA was prepared for Northern blot analysis with probe A2G2-9. In freshly isolated mid-pregnant mammary epithelial cells the level of SMC is undetectable, while that of β-casein is high (Fig. 3*A*). However, when the MEC are cultured on plastic with 10% fetal calf serum, SMC is detected at a high level, while β-casein, as expected, is reduced significantly. In the absence of serum on plastic SMC is expressed at a lower level, and β-casein is undetectable. In the presence of Matrigel and serum the level of SMC is low and that of β-casein is high. In Matrigel without serum the level of SMC is minimal and β-casein is unaffected. These data suggest that SMC levels are significantly enhanced by a factor in FCS, but greatly reduced by a factor present in the reconstituted basement membrane. Interestingly, although the protein level of SMC is negatively affected by the presence of Matrigel, the SMC transcript level is not decreased by culture on plastic or in Matrigel (Fig. 3*B*). The negative effect of Matrigel on SMC

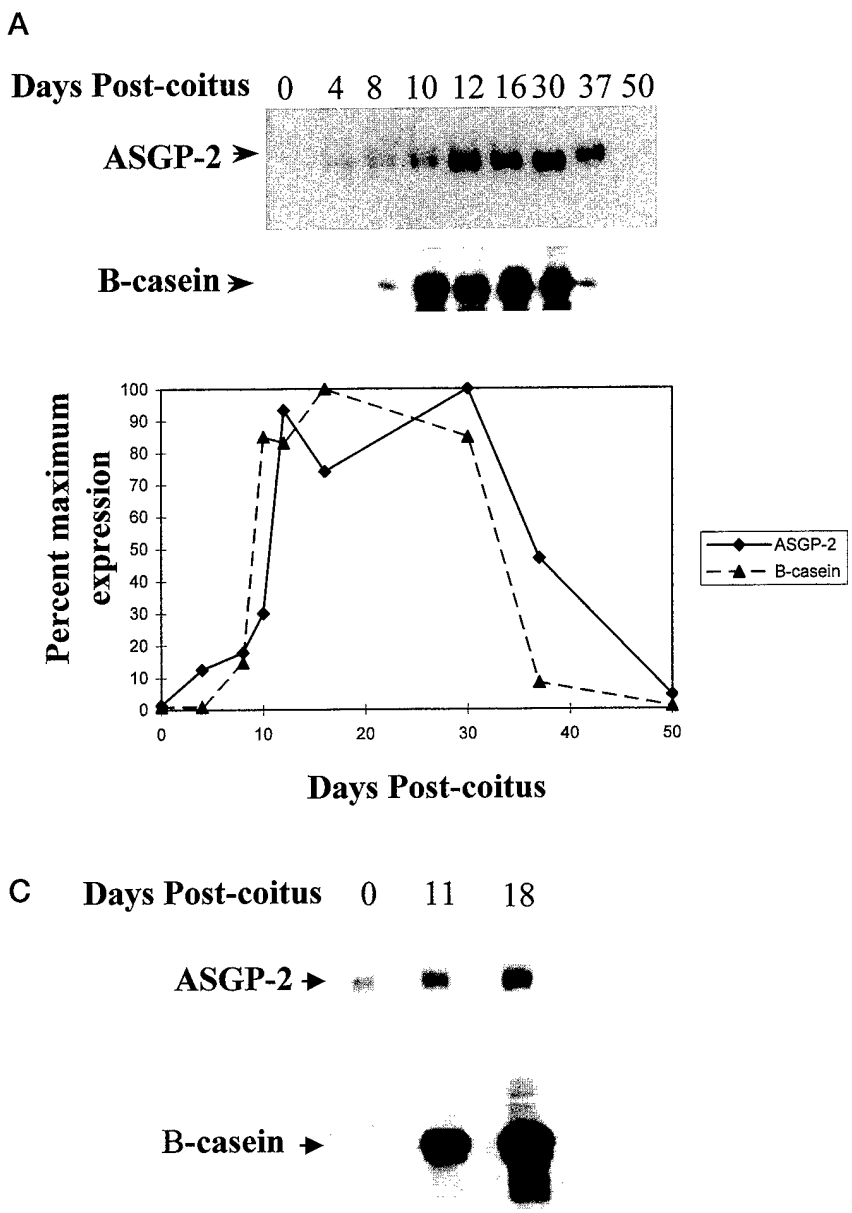


FIG. 1. **Expression of SMC and β-casein in the mammary gland and epithelial cells.** SDS-solubilized samples of virgin (day 0), pregnant (post-coitus days 4, 8, 10, 12, and 16), lactating (day 30), and involuting (days 37 and 50) mammary tissue were loaded (50 μg wet weight/lane) for SDS-PAGE as indicated at the *top* of each blot. *A,* immunoblots with anti-ASGP-2 or anti-β-casein antibodies are as indicated at the *left* of the figure. *B,* plot of percent maximum expression during mammary development. The bands from *A* were quantified by densitometry and the sample with the most intense staining was used as 100% (day 30 for SMC and day 16 for β-casein). *C,* SDS-solubilized samples of isolated mammary epithelial cells from virgin (day 0) and pregnant (days 11 and 18) rats were loaded (5 μg/lane total protein) for SDS-PAGE as indicated at the *top* of each blot. Immunoblots were performed with anti-ASGP-2 or anti-β-casein antibodies as indicated at the *left* of the figure.

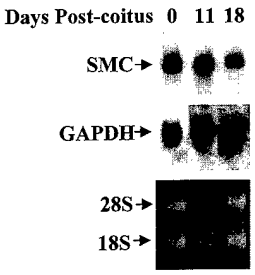


FIG. 2. **Northern blot analysis of SMC mRNA in normal rat mammary epithelial cells.** Total RNA was isolated from virgin (day 0) and pregnant (days 11 and 18) mammary epithelial cells, and 25 μg/lane were loaded for Northern blot analysis. Northern blots were probed with A2G2-9 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as indicated at the *left* of the figure.

protein levels is very different from its effect on β-casein, which is regulated by the ECM at the transcript level. These results suggest that SMC, under these conditions, is regulated post-transcriptionally, as it appears to be *in vivo*. Thus, this culture system is a useful *in vitro* model for determining factors that affect SMC expression in normal mammary gland.

SMC in MEC from Virgin Animals—β-Casein expression requires pregnancy or MEC culture under specific conditions that mimic the pregnant state (29, 30). Because the SMC transcript is present in abundance in MEC from virgin animals, these cells may already be primed for SMC production and awaiting the appropriate signal to synthesize the protein. Mammary epithelial cells were isolated from virgin rats and cultured on plastic with 10% serum to determine whether priming by pregnancy is necessary for the induction of SMC protein production or whether a change in the environment is sufficient for SMC biosynthesis. After 24 to 72 h in culture, the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In cultured virgin mammary epithelial cells, SMC reaches a maximal level in 24 h, and the level is maintained for at least 48 h (Fig. 4*A*). Thus, pregnancy is not required for the production of SMC. Induction of SMC biosynthesis appears to require only release of mammary epithelial cells from their environment, suggesting that in the virgin gland there is an inhibition of SMC synthesis which is released by removal of MEC or by the changes induced by pregnancy.

To determine whether extracellular matrix affects SMC in MEC from virgin animals similarly to that for MEC from preg-

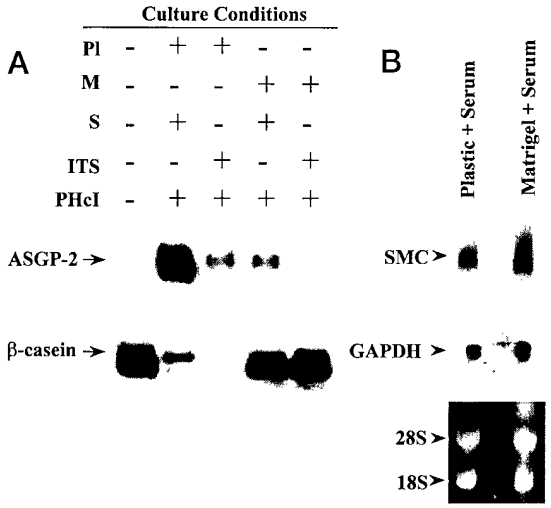


FIG. 3. **Effect of Matrigel on SMC expression in pregnant rat mammary epithelial cells.** Normal rat mammary epithelial cells were collected by collagenase digestion of day 11 pregnant rat mammary tissue followed by differential centrifugation. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence or absence of 10% FCS as indicated at the top of the figure. *A*, immunoblots of cultured pregnant mammary epithelial cells. SDS-solubilized cell lysates were loaded (5 μg/lane) for SDS-PAGE, and immunoblots were performed with monoclonal antibodies against ASGP-2 and β-casein as indicated at the *left* of the figure. *B*, Northern blot of cultured pregnant mammary epithelial cells. Total RNA was isolated from cultured epithelial cells and 25 μg/lane was loaded for Northern blot analysis. Northern blots were probed with A2G2-9. *Pl*, plastic; *M*, Matrigel; *S*, serum; *ITS*, insulin, transferrin, and selenium; *PHcI*, lactogenic hormones prolactin, hydrocortisone, and insulin.

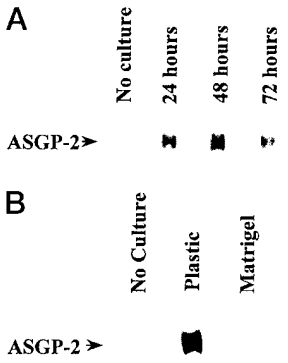


FIG. 4. **Expression of SMC in mammary epithelial cells from virgin rats.** Normal rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. MEC were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. Immunoblots were performed with anti-ASGP-2 antibodies as indicated at the *left* of the figure. *A*, induction of SMC expression in cultured virgin mammary epithelial cells. *B*, effect of Matrigel on SMC expression in cultured mammary epithelial cells from virgin rats.

nant animals, MEC from virgin rats were isolated and cultured in the presence of 10% serum either on plastic or embedded in Matrigel as described above. After 48 h in culture, cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 antibodies. In freshly isolated mammary epithelial cells, SMC is undetectable, as expected. When cultured on plastic, the level of SMC is high, and as in the cultured MEC from pregnant rats, the presence of Matrigel greatly inhibits the production of SMC (Fig. 4*B*). This inhibition occurs both when the cells are embedded in the Matrigel and when they are plated on top of it (data not shown), suggesting that one or more factors present in the Matrigel is involved in the reduction of

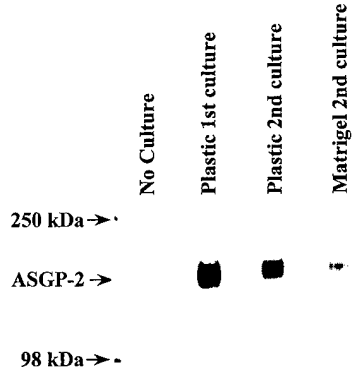


FIG. 5. **Effect of Matrigel on SMC levels in normal mammary epithelial cells already expressing SMC.** Virgin MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with 10% FCS. After 48 h, cells were removed from the plate with a non-enzymatic cell dissociation buffer. Half the cells were then replated in Ham's F-12 medium supplemented with 10% FCS either on plastic or embedded in Matrigel. After an additional 48 h in culture, cells were harvested and 5 μg total protein was subjected to immunoblot analysis with anti-ASGP-2 antibodies.

SMC levels in cultured mammary epithelial cells.

To study the effect of Matrigel on SMC in normal MEC already expressing the protein, MEC from virgin rats were prepared and cultured on plastic with 10% serum to induce high levels of SMC production. After 48 h, the cells were removed from the plate by non-enzymatic cell dissociation buffer and replated either on plastic or embedded in Matrigel for 48 h. Cleared cell lysates were subjected to immunoblot analysis with anti-ASGP-2 antibodies. After 48 h on plastic, SMC was present in abundance (Fig. 5). When cells were replated on plastic, the level of SMC remained high but at a slightly lower level than that of the first plating. The level of SMC in the cells embedded in Matrigel was significantly reduced. Thus, the level of SMC in normal MEC which already express the protein can be modulated by Matrigel.

Effect of Matrigel on SMC Levels in MAT-B1 Tumor Cells—Matrigel contains a number of components, including proteases which could digest cell surface proteins, thereby decreasing the levels of SMC in MEC cultured in it. To test this possibility, MAT-B1 ascites tumor cells, which express SMC abundantly on the cell surface, were cultured on plastic or embedded in Matrigel for 48 h. Cleared cell lysates and total RNA were prepared and subjected to immunoblot analysis with anti-ASGP-2 antibodies and Northern blot analysis with probe A2G2-9, respectively. Whether cultured on plastic or in Matrigel, the level of SMC was unchanged in the cultured MAT-B1 tumor cells (Fig. 6*A*), suggesting that proteolysis does not cause the decreased level of SMC in Matrigel. This finding is consistent with the observation that the level of ErbB2 in MEC cultured in Matrigel is unaffected (data not shown). Moreover, culture of rat tracheal epithelial cells (31) in Matrigel does not reduce their level of SMC, as would be expected from a proteolytic effect at the cell surface. The level of SMC message in the ascites tumor cells was also unaffected by the presence or absence of Matrigel (Fig. 6*B*). These data suggest that cell surface SMC is not degraded by proteases in Matrigel, and that regulation of SMC by Matrigel has apparently been altered significantly in these tumor cells.

SMC Synthesis in Virgin MEC—Regulation of SMC by Matrigel appears to be post-transcriptional in cultured MEC. Formally, this type of regulation may be by modification or degradation of SMC message, modulation of SMC message translation, or degradation of SMC protein. The steady state

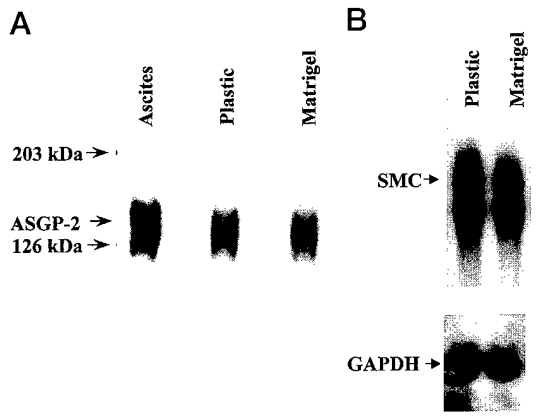


FIG. 6. **Effect of Matrigel on expression of SMC in 13762 MAT-B1 tumor cells.** Ascites MAT-B1 mammary adenocarcinoma cells were collected and plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. *A*, immunoblot with anti-ASGP-2 antibody. *B*, Northern blot of SMC message using probe A2G2-9 or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

levels of SMC message are unaffected by different culture conditions, suggesting that SMC message degradation is not the mechanism by which SMC is regulated in this system. To study how Matrigel affects SMC message translation, mammary epithelial cells were cultured either on plastic or embedded in Matrigel. After 24 h, the cells were metabolically labeled with [^{35}S]Cys +[^{35}S]Met for times ranging from 5 to 180 min. After labeling, the cells were washed, lysed, and immunoprecipitated with polyclonal anti-ASGP-2 antibody, which recognizes the SMC precursor as well as mature ASGP-2. It was necessary to immunoprecipitate the precursor because this product is intracellular and would not be affected by an extracellular protease. Immunoprecipitated SMC precursor was subjected to SDS-PAGE and fluorography, as was an aliquot of immunoprecipitated supernatant. To normalize for total protein, the bands for the SMC precursor and total protein were quantified by densitometry. The value for the SMC precursor was compared with that obtained for total protein. The SMC precursor band from cells cultured on plastic was easily visible by 20 min of labeling, while that from cells cultured in Matrigel was not visible until 90 min of labeling (Fig. 7*A*). When the ratio of SMC precursor to total labeled protein was calculated, the cells cultured on plastic synthesized SMC about eight times faster than those cultured in Matrigel (Fig. 7*B*). Thus, a factor in Matrigel reduces the mammary epithelial cells' ability to synthesize SMC, suggesting that SMC is regulated at the translational level in this system.

Effect of TGFβ on SMC Levels in Virgin MEC—Post-transcriptional regulation of milk protein gene expression and synthesis has not been widely explored. However, Robinson *et al.* (32) reported that TGFβ treatment can suppress the ability of pregnant mouse mammary tissue explants to synthesize and secrete casein. TGFβ treatment in their studies does not affect the level of casein mRNA, suggesting that it may regulate casein expression (synthesis) post-transcriptionally (32). TGFβ is also a component of Matrigel, and may therefore be a factor responsible for the decreased synthesis of SMC when MEC are cultured in it. To investigate the effect of TGFβ on SMC in normal virgin rat mammary gland, MEC were cultured in the presence or absence of 10% fetal bovine serum and 5 ng/ml TGFβ either on plastic or embedded in Matrigel. After 48 h the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibodies. MEC cultured on plastic or in Matrigel with serum have higher levels of SMC than those cultured in serum-free conditions, as shown previously. Also as expected, MEC cultured in Matrigel have lower levels of SMC than those cultured on plastic. However, the addition of TGFβ to MEC cultured in any of these conditions significantly reduces the level of SMC (Fig. 8), suggesting that TGFβ may be the factor in Matrigel involved in decreasing the levels of SMC when MEC are embedded in it.

Dose Response of SMC Suppression by TGFβ in Virgin MEC—The dose response of TGFβ-induced suppression of SMC synthesis was studied in normal virgin MEC. The MEC were isolated and cultured on plastic under serum-free conditions in the presence of increasing doses of TGFβ (0–20 ng/ml final concentration). After 48 h of culture, the cells were harvested and subjected to immunoblot analysis with anti-ASGP-2 monoclonal antibodies. Maximum levels of SMC are present when no TGFβ is added to the media, and as the level of TGFβ increases, the level of SMC decreases (Fig. 9). Thus, suppression of SMC synthesis by TGFβ in normal rat MEC is concentration-dependent. Furthermore, this response occurs at levels considered to be physiological.

Effect of TGFβ on SMC Transcript Levels in Virgin MEC—TGFβ suppresses the level of SMC in normal rat MEC in a manner similar to that of Matrigel. However, when SMC synthesis is suppressed by Matrigel, the level of SMC transcript is unaffected, suggesting a post-transcriptional mechanism of regulation in this system. To study the effect of TGFβ on SMC transcript levels in the normal rat, MEC were isolated and cultured on plastic in Ham's F-12 medium supplemented with insulin, transferrin, and selenium and 10 ng/ml TGFβ. After 48 h, the cells were harvested, total RNA was isolated, and 25 μg of total RNA was subjected to Northern blot analysis with probe A2G2-9. Interestingly, while the level of SMC protein is significantly reduced by the presence of TGFβ, the level of SMC transcript was relatively unaffected by the presence of TGFβ under these conditions (Fig. 10). These results support a role for TGFβ in the post-transcriptional regulation of SMC expression in normal rat mammary tissue.

DISCUSSION

SMC is developmentally regulated in normal rat mammary gland. *In vivo*, the protein is present at a low level in virgin gland and increases dramatically during mid-pregnancy, as does β-casein. However, SMC reaches maximum levels about 2 days later than β-casein, suggesting that its expression has a different regulatory mechanism. Interestingly, the SMC transcript is present at similar levels in virgin and pregnant mammary gland, in contrast to the expression of β-casein transcript, which only becomes detectable around day 6 of pregnancy (6). The expression pattern of SMC *in vivo*, where the level of protein does not directly correspond to the level of transcript, suggests a post-transcriptional mechanism of regulation for this protein.

To study the mechanism of SMC regulation and the factors which may influence SMC expression in normal mammary gland, we utilized primary mammary epithelial cell culture. Primary culture of mammary epithelial cells has been used to elucidate the regulatory mechanisms of several milk proteins. These systems are thought to mimic the *in vivo* state and are useful because the level of differentiation/functionality can be maintained or manipulated. For example, in the presence of Matrigel and lactogenic hormones (insulin, hydrocortisone, and prolactin), isolated mammary epithelial cells will form round alveolar structures with closed lumens and vectorially secrete milk proteins (4, 28, 32–34). On plastic these cells form squamous epithelial monolayers and are considered undifferentiated because milk proteins are not synthesized (5).

Expression of β-casein in isolated mammary epithelial cells is dependent on the presence of extracellular matrix compo-

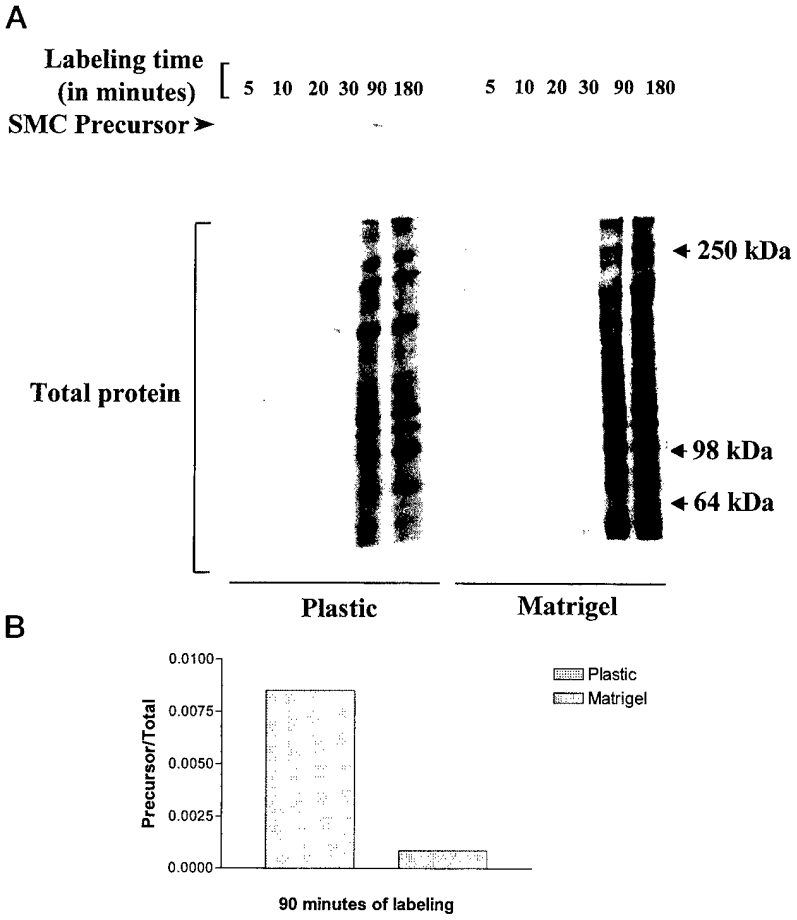


FIG. 7. **Effect of Matrigel on SMC precursor synthesis in normal rat mammary epithelial cells.** Rat mammary epithelial cells were collected by collagenase digestion of virgin rat mammary tissue. Epithelial cells were plated in Ham's F-12 media either on plastic or embedded in Matrigel in the presence of 10% FCS. After 24 h, cells were labeled for the times indicated with [^{35}S]Met + [^{35}S]Cys. Cells were lysed in 2% SDS, diluted into a Triton X-100 buffer, and immunoprecipitated with anti-ASGP-2 polyclonal antibody and protein A-agarose. Immunoprecipitates were subjected to SDS-PAGE and fluorography. *A*, fluorogram of immunoprecipitated SMC precursor and total protein. *B*, difference in SMC translation rate in the presence or absence of Matrigel. The SMC precursor band and its corresponding total protein lane from the 90-min labeling sample were quantified by densitometry. The ratios of SMC precursor to total protein were plotted in *B*.

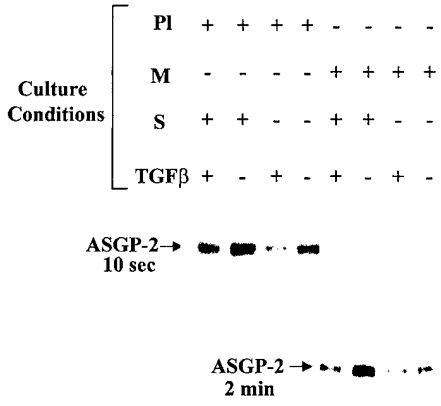


FIG. 8. **Effect of TGFβ on SMC protein levels in normal MEC.** Normal rat MEC were isolated and cultured either on plastic or embedded in Matrigel in the presence or absence of 10% FCS and 5 ng/ml TGFβ as indicated at the *top* of the figure. Cell were lysed and the lysates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12. Note: exposure time is indicated at the left of the figure. Samples from cells cultured in Matrigel were exposed for 2 min as compared with 10 s for cells cultured on plastic in order to detect a significant amount of staining. *Pl*, plastic; *M*, Matrigel; *S*, serum.

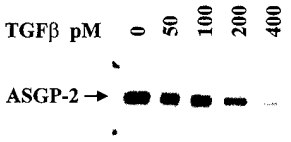


FIG. 9. **Dose response of SMC to TGFβ in normal rat MEC.** Normal rat MEC were isolated and cultured on plastic under serum-free conditions with varying concentrations of TGFβ as indicated at the *top* of the figure. Cell lysates were subjected to immunoblot analysis with anti-ASGP-2 mAb 4F12.

nents (or basement membrane). In the presence of a reconstituted basement membrane (Matrigel), mammary epithelial cells from pregnant mice and virgin rats can be induced to express β-casein (5, 29). There are several major differences in the behavior of β-casein and SMC in primary MEC cultures in the presence or absence of basement membrane. 1) SMC is found at the highest levels when pregnant rat MEC are cultured on plastic and at very low levels when the cells are embedded in Matrigel. β-Casein is found at maximal levels when pregnant rat MEC are embedded in Matrigel and only at minimal levels when the cells are cultured on plastic. 2) SMC transcript levels are not affected by MEC culture on plastic or in Matrigel, while β-casein transcript levels have been shown to be low (or non-existent) when MEC are cultured on plastic and high when MEC are embedded in Matrigel (35). 3) SMC protein production can be induced in MEC from virgin rats within hours of culture on plastic, suggesting that MEC do not need to be primed by pregnancy to be capable of synthesizing SMC. β-Casein expression, on the other hand, requires MEC priming by pregnancy or long-term culture under specific conditions for expression (29, 30). Primary culture of normal rat MEC does seem to mimic the *in vivo* state with respect to SMC expression (as well as β-casein expression, as shown by others). Furthermore, these data suggest that SMC is regulated by a post-transcriptional mechanism.

There are also several differences in SMC expression in normal MEC and the 13762 rat mammary adenocarcinoma, in which SMC was first identified. The 13762 rat mammary adenocarcinoma ascites cells express SMC at a level at least 100-fold higher than that in normal lactating mammary gland (8).

1) Unlike normal MEC, which express SMC at high levels upon removal from the animal, the MAT-B1 tumor cells significantly reduce SMC expression within 48 h during culture after removal from the animal. 2) Culture in Matrigel does not seem to affect SMC levels in MAT-B1 tumor cells, either at the level of protein or transcript. In normal MEC, however, Matrigel reduces SMC levels in newly cultured normal MEC and in MEC that already express SMC. It appears that regulation of SMC expression in MAT-B1 tumor cells has been disrupted compared with that of normal MEC. In the MAT-B1 cells the transcript and protein are both synthesized equally well on plastic or in Matrigel, while in normal MEC the transcript is made but protein synthesis is reduced significantly in Matrigel. We have no evidence to suggest that SMC from the tumor cells is different from SMC from normal cells. The maintenance of SMC in the presence of Matrigel in the tumor confirms that the reduction of SMC seen in normal MEC is not due to the presence of a protease in the Matrigel. More likely, the tumor cells have lost their responsiveness to Matrigel.

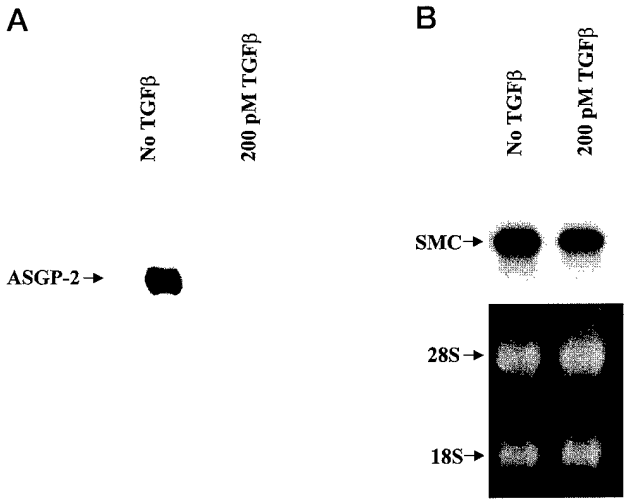


FIG. 10. **Effect of TGFβ on SMC transcript levels in normal MEC.** Normal rat MEC were isolated and cultured on plastic in serum-free medium in the presence or absence of 10 ng/ml TGFβ. Total RNA was isolated and 25 μg of total RNA subjected to Northern blot analysis with probe A2G2-9. *A,* immunoblot with mAb 4F12 indicates a large decrease in the level of SMC when MEC are cultured in the presence of TGFβ. *B,* Northern blot with probe A2G2-9 indicates no significant change in SMC message when MEC are cultured in the presence of TGFβ.

Post-transcriptional regulation of milk proteins has not received substantial attention. TGFβ can decrease casein production in mammary organ explants without affecting its transcript level (32). SMC synthesis in normal rat MEC is also suppressed by TGFβ with no effect on its transcript level. This result suggests that TGFβ may be responsible for regulating both casein and SMC post-transcriptionally in the normal developing mammary gland. Some other post-transcriptional regulation mechanisms have also been described. For example, it has been reported that β-casein transcript is stabilized by the presence of prolactin (36). WAP, a late milk protein, requires MEC culture in a three-dimensional matrix and a hollow alveolar structure with a closed lumen for expression (6). However,

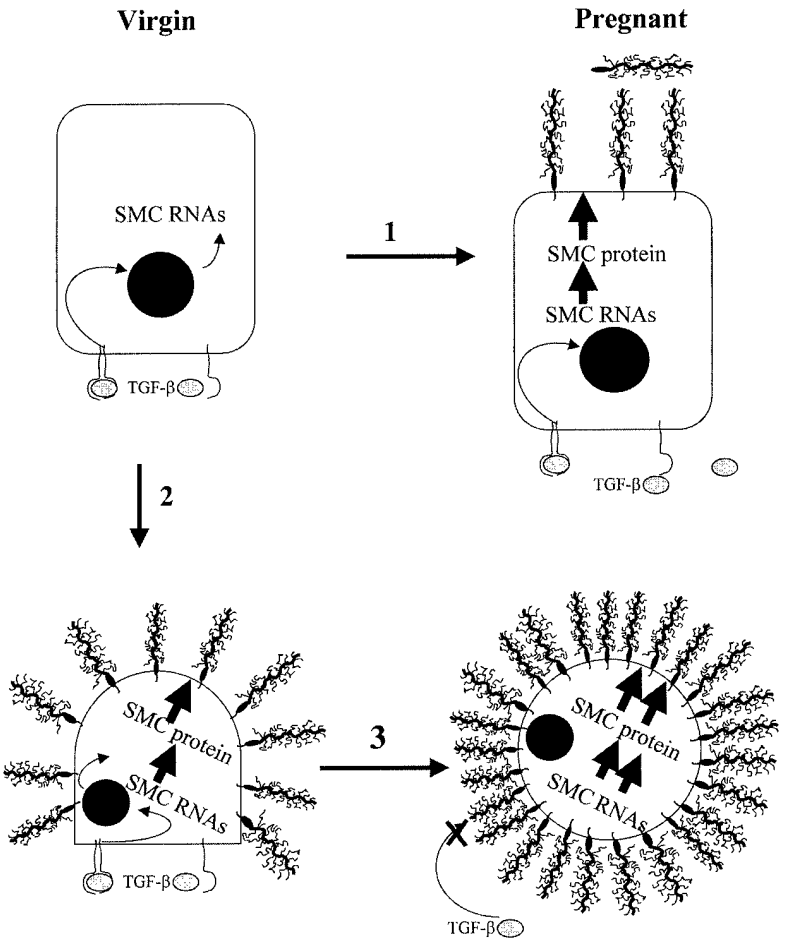


FIG. 11. **Model for regulation of SMC expression in normal mammary epithelial cells and its disruption in mammary tumor cells.** In normal virgin MEC SMC RNA is present at high levels, but there is some modification or inhibitor regulated by TGFβ that blocks translation of the SMC message. Thus, SMC protein is present at very low levels in virgin MEC. *1,* during pregnancy, as the ECM, hormone, and growth factor milieu changes, the TGFβ effect is overridden or TGFβ is down-regulated, allowing for biosynthesis of SMC protein. *2,* at some point in neoplastic transformation, TGFβ responsiveness is lost, SMC begins to be overexpressed, and the cell loses polarity. *3,* as the tumor becomes more aggressive and SMC is grossly overexpressed, cell-cell and cell-matrix interactions are disrupted and there is loss of recognition by the immune system, allowing for metastasis of the aggressive tumor. The mechanism by which the TGFβ effect is overcome during pregnancy and disrupted in the 13762 ascites tumor cells is currently under investigation.

there is no difference between WAP transcription on plastic or on basement membrane, suggesting another post-transcriptional regulatory mechanism (6).

SMC, which is apparently the rat homolog of human MUC4, is expressed in other tissues besides mammary gland. SMC has a similar tissue distribution to that reported for MUC4. SMC is constitutively expressed in normal rat trachea, colon goblet cells, and small intestinal Paneth cells (8, 17, 37, 38). Human MUC4 gene expression has also been in numerous epithelial tissues, including trachea, small and large intestine, and cervix (23). SMC expression in the uterus appears to be under the control of the ovarian hormones estrogen and progesterone at the transcript level (22). Suppression of SMC synthesis by TGFβ is probably not the only mechanism for regulating its expression in normal mammary gland. The complex tissue distribution and expression pattern of SMC in normal rat tissues suggests that it has multiple and complex regulatory mechanisms.

Regulation of milk proteins is achieved through complex interactions of hormones, growth factors, and extracellular matrix components (1–5). Many studies have been done to sort out the regulatory pathways for β-casein and WAP, and from these, many specific factors have been shown to play roles in complex cascades of events leading to functional differentiation of mammary tissue. For example, β-casein expression depends largely on the lactogenic hormone prolactin and the extracellular matrix component laminin (7, 39). β-Casein expression does not require cell-cell contact or closed alveolar structures, and its expression is inhibited by the growth factors TGFβ and EGF (4, 5, 40–42). WAP, on the other hand, requires cell-cell contact in the formation of closed alveolar structures in an ECM, the glucocorticoid hydrocortisone, and the down-regulation of the growth factor TGFα (4, 39, 43). SMC is at least partially regulated by TGFβ in normal mammary gland. Studies are currently underway to determine other factors or combinations of factors which may regulate SMC levels in normal mammary tissue.

The data presented here suggest that SMC, a product of differentiated mammary tissue, appears to be regulated post-transcriptionally in normal mammary tissue. There are several ways protein production can be regulated post-transcriptionally. These include stability of the transcript, changes in the rate of message translation, and changes in the rate of protein degradation or turnover. The steady state levels of SMC transcript were unaffected either by developmental state *in vivo* or by culture conditions *in vitro*, suggesting that SMC transcript stability in these conditions is unchanged. Because the level of SMC protein is significantly reduced in Matrigel, it appears that SMC protein is turning over in the cultured cells. Moreover, when cells cultured on plastic or Matrigel are metabolically labeled and SMC precursor is immunoprecipitated and quantified relative to total labeled protein, SMC is translated at ~8-fold higher rate on plastic than in Matrigel. Changes in SMC protein stability under these culture conditions are being investigated. However, taken together these results suggest that SMC biosynthesis is post-transcriptionally regulated in normal MEC by a factor present in Matrigel, most likely TGFβ.

Other explanations could be proposed for differences in SMC transcript and protein expression in the virgin mammary gland and for the apparent decrease of SMC levels in the presence of Matrigel and TGFβ. One possibility is that the monoclonal antibody 4F12 (which recognizes an epitope in the N-terminal 53 amino acids of ASGP-2) used to detect SMC may not recognize alternative splice forms of ASGP-2 or forms that have post-translational modification, such as differential phosphorylation or glycosylation. However, other monoclonal antibodies against ASGP-2 that recognize other epitopes in the central portion of ASGP-2 (more C-terminal) yield similar results to those presented (data not shown), suggesting that these are unlikely possibilities. In addition, previous attempts to demonstrate alternative splice forms of SMC in normal rat tissues or the ascites tumor cells have been unsuccessful (8). Another possibility is that there is proteolytic cleavage of SMC producing secreted forms of SMC, which are not detected. We have reported detection of secreted SMC from lactating mammary tissue by serial immunoprecipitation with polyclonal antibodies against the C-terminal peptide followed by immunoprecipitation with polyclonal antibodies against total SMC to precipitate any SMC not recognized by the C-terminal antibody (8). Furthermore, we can also detect similar ratios of the membrane bound and secreted forms of SMC in the cultured virgin mammary epithelial cells used in these studies (data not shown). Therefore, although these possibilities cannot be entirely ruled out, our data strongly suggest that the antibodies used in the studies reported here do recognize all forms of SMC and that the apparent decrease in the level of SMC in the presence of Matrigel and TGFβ is due to a real decrease in SMC protein levels under these conditions.

From these studies we can propose the following model. Virgin rat mammary epithelial cells are primed for SMC production by the continual presence of SMC transcript, but the transcript is not translated because of some RNA modification or translational inhibitor under the control of the TGFβ pathway (Fig. 11). As pregnancy proceeds and the growth factor, hormone, and ECM milieu change, this modification or inhibition is altered, the suppression of SMC synthesis by TGFβ is released and SMC synthesis can occur. Upon removal of MEC from the animal the repression is also relieved, presumably by removing TGFβ from the epithelial cell environment, allowing biosynthesis of SMC under the culture conditions we have described.

In the MAT-B1 tumor cells this level of regulation appears to have been disrupted, and this, in combination with the 5-fold amplification of the gene and overexpression of the transcript contributes to its gross overexpression in this tumor cell line (15) (Fig. 11). TGFβ may be involved in keeping the expression of this protein in check in the normal animal. Loss of TGFβ responsiveness is a significant factor in tumor progression, and may be involved in the overexpression of SMC in the MAT-B1 ascites tumor cells. Loss of TGFβ suppression thus may contribute to SMC overexpression in these cells, along with other factors. The growth factor-like properties of SMC may then contribute to uncontrolled proliferation of these cells, while the mucin subunit contributes to protection from the immune system and loss of adhesion. In summary, SMC is an unusual post-transcriptionally regulated milk membrane protein, whose overexpression contributes properties conductive to tumor progression. Elucidation of its regulatory mechanism by TGFβ and other factors in normal developing mammary gland and its disruption in the 13762 tumor cells will give further insight into both normal developmental processes and tumor progression. Since translational regulation might be involved, investigation of factors regulating translation (44) should be of interest, including the 0.3-kilobase 3′-untranslated region of the transcript.

Acknowledgment—We thank Dr. Charlotte Kaetzel (University of Kentucky, Lexington, KY) for generously providing the anti-β-casein mouse monoclonal antibody used for immunoblots.

REFERENCES

1. Topper, Y. J., and Freeman, C. S. (1980) *Physiol. Rev.* **60,** 1049–1106
2. Sakakura, T., Ishihara, A., and Yatini, R. (1982) in *Regulatory Mechanisms in Breast Cancer* (Lippman, M., and Diskson, R., eds) pp. 383–400, Kluwer, Hingham, Boston

3. Daniel, C. W., and Silberstein, G. B. (1987) in *The Mammary Gland: Development, Regulation and Function* (Neville, M., and Daniel, C., eds) pp. 3–36, Plenum Press, New York
4. Chen, L. H., and Bissell, M. J. (1989) *Cell Regul.* **1,** 45–54
5. Streuli, C. H., Bailey, N., and Bissell, M. J. (1991) *J. Cell Biol.* **115,** 1383–1395
6. Lin, C. Q., and Bissell, M. J. (1993) *FASEB J.* **7,** 737–743
7. Streuli, C. H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A. P. N., Roskelley, C., and Bissell, M. J. (1995) *J. Cell Biol.* **129,** 591–603
8. Rossi, E. A., McNeer, R., Price-Schiavi, S. A., Van den Brande, J. M. H., Komatsu, M., Thompson, J. F., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1996) *J. Biol. Chem.* **271,** 33476–33485
9. Sherblom, A. P., Buck, R. L., and Carraway, K. L. (1980) *J. Biol. Chem.* **255,** 783–790
10. Sherblom, A. P., and Carraway, K. L. (1980) *J. Biol. Chem.* **255,** 12051–12059
11. Carraway, K. L., and Spielman, J. (1986) *Mol. Cell. Biochem.* **72,** 109–120
12. Carraway, K. L., and Hull, S. R. (1991) *Glycobiology* **1,** 131–138
13. Hull, S. R., Sheng, Z., Vanderpuye, O. A., David, C., and Carraway, K. L. (1990) *Biochem. J.* **265,** 121–129
14. Sheng, Z., Wu, K., Carraway, K. L., and Fregien, N. (1992) *J. Biol. Chem.* **267,** 16341–16346
15. Wu, K., Fregien, N., and Carraway, K. L. (1994) *J. Biol. Chem.* **269,** 11950–11955
16. Sheng, Z., Hull, S. R., and Carraway, K. L. (1990) *J. Biol. Chem.* **265,** 8505–8510
17. McNeer, R. R., Price-Schiavi, S. A., Komatsu, M., Fregien, N., Carraway, C. A. C., and Carraway, K. L. (1997) *Front. Biosci.* **2,** 449–459
18. Komatsu, M., Carraway, C. A. C., Fregien, N. L., and Carraway, K. L. (1997) *J. Biol. Chem.* **272,** 33245–33254
19. Howard, S. C., Hull, S. R., Huggins, J. W., Carraway, C. A. C., and Carraway, K. L. (1982) *J. Natl. Cancer Inst.* **69,** 33–40
20. Carraway, K. L., III, and Cantley, L. C. (1994) *Cell* **78,** 5–8
21. Juang, S.-H., Carvajal, M. E., Whitney, M., Liu, Y., and Carraway, C. A. C. (1996) *Oncogene* **12,** 1033–1042
22. McNeer, R. R., Carraway, C. A. C., Fregien, N., and Carraway, K. L. (1998) *J. Cell. Physiol.* **176,** 110–119
23. Nollet, S., Moniaux, N., Maury, J., Petitprezk D., Degand, P., Laine, A., Porchet, N., and Aubert, J.-P. (1998) *Biochem. J.* **332,** 739–748
24. Porchet, N., Nguyen, V. C., Dufosse, J., Audie, J. P., Guyonnet Duperat, V., Gross, M. S., Denis, C., Degand, P., Bernheim, A., and Aubert, J. P. (1991) *Biochem. Biophys. Res. Commun.* **175,** 414–422
25. Carraway, K. L., Doss, R. C., Huggins, J. W., Chesnut, R. W., and Carraway, C. A. C. (1979) *J. Cell Biol.* **83,** 529–543
26. Emerman, J. T., Enami, J., Pitelka, D. R., and Nandi, S. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 4466–4470
27. Lee, E. Y.-H., Parry, G., and Bissell, M. J. (1984) *J. Cell Biol.* **98,** 146–155
28. Barcellos-Hoff, M. H., Aggeler, J., Ram, T. G., and Bissell, M. J. (1989) *Development (Camb.).* **105,** 223–235
29. Darcy, K. M., Black, J. D., Hahm, H. A., and Ip, M. M. (1991) *Exp. Cell Res.* **196,** 49–65
30. Robinson, G. W., McKnight, R. A., Smith, G. H., and Hennighausen, L. (1995) *Development* **121,** 2079–2090
31. McNeer, R. R., Huang, D., Fregien, N., and Carraway, K. L. (1998) *Biochem. J.* **330,** 737–744
32. Robinson, S. D., Roberts, A. B., and Daniel, C. W. (1993) *J. Cell Biol.* **120,** 245–251
33. Li, M. L., Aggeler, J., Farson, D. A., Hatier, C. J., Hassell, C., and Bissell, M. J. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 136–140
34. Aggeler, J., Ward, J., Blackie, L. M., Barcellos-Hoff, M. H., Streuli, C. H., and Bissell, M. J. (1991) *J. Cell Sci.* **99,** 407–417
35. Lee, E. Y.-H., Lee, W.-H., Katzel, C. S., Parry, G., and Bissell, M. J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 1419–1493
36. Mercier, J.-C., and Vilotte, J.-L. (1993) *J. Dairy Sci.* **76,** 3079–3098
37. Rossi, E. A. (1996) *Characterization of the Tissue and Cell Specific Expression of Ascites Sialoglycoprotein-2 and Its Association with p185^{neu}.* Ph. D. Thesis, University of Miami
38. McNeer, R. R. (1997) *Expression and Characterization of an ASGP-1/ASGP-2 Sialomucin Complex in Normal Tissues.* Ph. D. Thesis, University of Miami
39. Pittius, C. W., Sankaran, L., Topper, Y. J., and Hennighausen, L. (1988) *Mol. Endocrinol.* **2,** 1027–1032
40. Taketani, Y., and Oka, T. (1983) *FEBS Lett.* **152,** 256–260
41. Taketani, Y., and Oka, T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1646–1649
42. Mieth, M., Boehmer, F. D., Ball, R., Groner, B., and Grosse, R. (1990) *Growth Factors* **4,** 9–15
43. Lin, C. Q., Dempsey, P. J., Coffey, R. J., and Bissell, M. J. (1995) *J. Cell Biol.* **129,** 1115–1126
44. Jackson, R. J., and Standart, N. (1990) *Cell* **62,** 15–24